

University of Alberta

**Regulation of the Na^+/H^+ Exchanger Isoform I by
Phosphorylation of Ser⁷⁶⁶, Ser⁷⁷⁰, & Ser⁷⁷¹**

By

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*I thank my soon-to-be husband, family, and friends for their
immeasurable support throughout my academic career. Their continued
strength inspires me to pursue all my dreams without fear.*

Abstract

Regulation of the Na^+/H^+ exchanger isoform I (NHE1) by phosphorylation was investigated. Specific groups of serine and threonine residues in the C-terminal tail of NHE1 were mutated to alanine residues. The proteins were expressed in NHE-deficient AP-1 cells and characterized. All mutants had proper expression, localization, and activity relative to wildtype NHE1. Sustained intracellular acidosis was used to activate NHE1 via a $\text{Raf} \rightarrow \text{MEK1} \rightarrow \text{ERK2} \rightarrow \text{p90}^{\text{RSK}}$ pathway that could be blocked with the MEK inhibitor U0126. The activity of mutants S^{693}A , $\text{T}^{718}\text{A-S}^{723/26/29}\text{A}$, and $\text{T}^{779}\text{A-S}^{785}\text{A}$ was stimulated similar to wildtype NHE1. Mutant $\text{Ser}^{766/70/71}\text{Ala}$ lost the stimulatory effect. Immunoprecipitation of NHE1 showed that loss of stimulation corresponded to a loss of sustained acidosis-mediated NHE1-phosphorylation. Based on these results, one or all of residues Ser^{766} , Ser^{770} , and Ser^{771} are implicated in an important regulatory role activating NHE1 through an ERK-dependent pathway.

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Abbreviations

α_{1A} -AR	α_{1A} -adrenoreceptor
12CA5	Anti-HA antibody
Akt/PKB	Protein kinase B
Amp	Ampicillin
Amp ^R	Ampicillin resistance
AngII	Angiotensin II
AT ₁ /AT ₂	Angiotensin receptor subtypes 1 and 2
BCECF	2',7'-bis(carboxyethyl-5 (and 6)-carboxy) fluorescein
BCECF-AM	2',7'-bis(carboxyethyl-5 (and 6)-carboxy) fluorescein acetoxymethyl ester
BGS	Bovine growth serum
BSA	Bovin serum albumin
CAII	Carbonic anhydrase II
CABG	Coronary artery bypass graft
CaM	Calmodulin
CaM kinase II	Ca ²⁺ /calmodulin-dependent protein kinase II
CFTR	Cystic fibrosis transmembrane regulator
CHO cells	Chinese hamster ovary cells
CHP1	Calcineurin homologous protein 1
C-terminal	Carboxy terminal
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor

EL	Extracellular loop
ERK	Externally regulated kinase
ERM	Ezrin, radixin, and moesin
ESCAMI study	Evaluation of the Safety & Cardioprotective effects of eniporide in Acute Myocardial Infarction
ET-1	Endothelin
EXPEDITION study	Na ⁺ /H ⁺ Exchange inhibition to Prevent coronary Events in acute cardiac conDITIONs study
FPLC	Fast protein liquid chromatography
G418	Geneticin
GAM	Goat anti-mouse
GPCR	G-protein coupled receptor
GUARDIAN study	GUARd During Ischemia Against Necrosis study
HRP	Horse radish peroxidase
I2	Inhibitor 2
IL	Intracellular loop
IP	Immunoprecipitation
IP ₃	inositol 1,4,5-triphosphate
LPA	Lysophosphatidic acid
MALDI	matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
MEK	MAP-ERK kinase
MEM	Minimum essential medium
MI	Myocardial infarction
MS	Mass spectrometry
NCX	Sodium/Calcium exchanger

Nha	Sodium/Proton antiporter
NHE	Sodium/Proton exchanger
NIK	Nck-interacting kinase
NMR	Nuclear magnetic resonance
NRVM	Neonatal rat ventricular myocytes
N-terminal	Amino terminal
OA	Okadaic acid
OT-IMAC	Open tubular immobilized metal ion affinity chromatography
p90 ^{RSK}	Ribosomal protein S6 kinase
PAR-1	Protease-activated receptor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH _i	Intracellular pH
PIP ₂	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl Fluoride
PP1/2A	Protein phosphatase 1 and 2A
RoR	Rate of Recovery
RSV	Rous sarcoma virus
Tb	Thrombin
TBS	Tris
TM	Transmembrane
VSMC	Vascular smooth muscle cells
Zn	Zinc

Chapter I

Introduction

1. Intracellular pH in mammalian cells

Intracellular pH (pH_i) is finely controlled in mammalian cells as many basic systems depend on it. Proteins require a specific pH for optimal functioning, normal and pathological gene expression is regulated by pH_i , and ion concentrations are affected since many channels and pumps are involved in H^+ transport and are affected by pH_i (53, 66, 105). To regulate pH_i mammalian cells express a number of proteins at their plasma membrane that transport H^+ and HCO_3^- into and out of the cell (104). Important participants in the precise system of pH_i regulation include the Na^+/H^+ exchanger (NHE) family of proteins, and various bicarbonate transporters and exchangers.

Proton efflux is a major factor in pH_i maintenance as normal cellular metabolism generates an intracellular acid load. NHE proteins are activated by acidic cellular conditions to catalyze the electroneutral exchange of one extracellular sodium ion for one intracellular proton and as such, constitute a key component in the protection against cellular acidosis. This exchange process is dependent on extracellular Na^+ and ATP, although the protein does not directly consume metabolic energy (1, 89). The family of NHE proteins includes 9 isoforms each with unique tissue and cellular distribution, inhibitor sensitivities, regulatory elements, and ensuing physiological roles. NHE1, the first isoform identified, is distinct in that it is ubiquitously expressed in all mammalian cells and plays a house-keeping role.

2. Na^+/H^+ exchanger family

2.1. NHE1

As early as 1982 Pouyssegur *et al* had identified a " Na^+/H^+ exchange system" that played an important role in regulation of pH_i and had a major influence on growth factor action (102). Further studies identified biochemical characteristics of this system, for example, the addition of external Na^+ to a cell caused the release of internal H^+ with a 1:1 stoichiometry. The exchange of sodium and protons was also amiloride sensitive, and this exchange system existed on the plasma membrane of cells (96). In 1984 Pouyssegur and his group developed a H^+ -suicide technique that allowed them to select for a cell line deficient in Na^+/H^+ exchange activity. This proved to be a useful model for identifying the actual role(s) of this exchanger (102).

In 1989 Sardet *et al* cloned the first Na^+/H^+ exchanger isoform, NHE1, and based on the cDNA sequence the amino acid sequence was predicted (119). Since the initial cloning of NHE1, many studies have looked at protein expression and localization, mechanistic and regulatory features, and physiological roles for the protein.

NHE1 is ubiquitously expressed in all mammalian cells and is considered the "housekeeping" isoform. After its initial discovery, NHE1 was also identified as the predominant isoform expressed in the myocardium and therefore is considered the cardiac specific isoform (37). NHE1 cellular localization varies

depending on the cell type. In fibroblasts where NHE1 is involved in migration and anchoring, the protein is predominantly localized in lamellipodia (30, 31). NHE1 is distributed to the basolateral membrane of epithelial cells (17, 89), and within myocardial tissue it is concentrated in the intercalated disks and along the transverse tubular system (99).

NHE1 is the most inhibitor sensitive isoform (79). Amiloride was the first drug identified as an NHE inhibitor although it does have an inhibitory effect on various other channels and exchangers. Amiloride is thought to bind to the external Na^+ binding site since inhibitory potency is reduced in high Na^+ concentrations (79). There have since been a number of drugs developed that are more selective for NHE1 including the benzoylguanidine inhibitors. This group of inhibitors include HOE694, cariporide, and eniporide; the latter two have been studied in the clinical setting (134, 157).

The physiological roles of NHE1 have been thoroughly studied, and have proven to be diverse. The most basic role of NHE1 is in pH_i and cell volume control, but these factors can have wide ranging implications on numerous cellular processes. NHE1 is involved in normal processes such as cell proliferation, growth, migration, and apoptosis, and pathological processes such as cancer cell invasion and heart failure (48, 57).

2.2. NHE2-NHE9

Cloning of NHE2 first occurred in the rat where the isoform was identified as having an amino acid sequence that shared 42% identity with NHE1 (148), but since that discovery in depth studies on the human protein have provided us with a more thorough understanding of this isoform (77). NHE2 is an apical membrane protein that is expressed in the epithelial tissue of the intestinal tract and kidney, skeletal muscle, and the testis (77). Within the digestive tract NHE2 is quite prominent with strong expression in the jejunum, ileum, and colon (20). Specific expression in renal tissue has been localized to the cortical thick ascending limbs, distal convoluted tubules, connecting tubules, and the macula densa cells (23, 98). This isoform has similar inhibition constants for both amiloride and non-amiloride compounds as that observed for NHE1 (156). NHE2 is expressed in the parietal cells of the gastric epithelium, and the *Nhe2*^{-/-} mouse shows that although NHE2 is not required for acid secretion by these cells, it is essential for their long-term viability (124). Overall, NHE2 appears to be involved in several secretory processes.

The Na⁺/H⁺ exchanger isoforms 3 and 4 were both identified in 1992 and were found to have 39% and 42% amino acid identity to NHE1 respectively (90). Furthermore, both NHE3 and NHE4 were identified as having significant levels of expression in the kidney and gastrointestinal tract. In the gastrointestinal tract NHE3 has higher expression in the intestine while NHE4 appears to be localized

predominantly in the stomach. In renal tissue NHE3 has been identified as an apical membrane protein in the proximal tubule and thick ascending limb (3) while the NHE4 isoform has basolateral epithelial distribution and is found specifically in the inner medulla of the kidney (20, 101). More recently, both isoforms have also been identified in the submandibular gland in rats, with NHE3 having apical expression in duct cells and NHE4 having basolateral expression in acinar and ducts cells (88). Both NHE3 and NHE4 are relatively resistant to amiloride and non-amiloride inhibitors (21, 156). NHE4 requires hyperosmolarity for activation (18, 19). Mouse knockout models of NHE3 and NHE4 show that NHE3 is the major absorptive Na^+/H^+ exchanger in the kidney and intestine, while NHE4 is involved in regulating normal levels of gastric acid secretion (41, 123).

Studies on NHE5 have revealed that its expression is restricted to the brain in both rats and humans. Sequence analysis demonstrated that NHE5 has 39% amino acid identity to NHE1, but also 53% identity to NHE3. In addition to having similar primary structure to NHE1, NHE5 also has similar inhibitor sensitivities to NHE3 (5, 9). Overall, NHE5's restricted expression in the brain suggests a specialized role for the isoform, but as of yet no physiological role has been elucidated.

Na^+/H^+ exchanger isoforms 6-9 are unique to NHE1-5 in that rather than being localized on the plasma membrane of cells, these isoforms are localized to the membranes of the Golgi and post-Golgi endocytic compartments. Primarily,

NHE6 is expressed in the early recycling endosomes, NHE7 in the trans-Golgi network, NHE8 in the mid- to trans-Golgi, and NHE9 in the late recycling endosomes, although there is some overlap in distribution. It is likely that these NHE isoforms contribute to pH maintenance of these intracellular organelles (86).

3. Structural features of the Na^+/H^+ exchanger

3.1. Structural domains

The human NHE1 cDNA predicts an 815 amino acid protein. The first 500 residues are predicted to be transmembrane (TM) spanning segments, and the remaining 315 residues are thought to constitute an intracellular regulatory domain (119). Using cysteine-accessibility analysis Wakabayashi *et al* confirmed a twelve TM spanning N-terminal domain that differed from the initial Kyte-Doolittle model that had been predicted using hydropathy analysis (64, 144). The Kyte-Doolittle model did predict twelve transmembrane regions, Wakabayashi's model also predicted one extracellular and two intracellular membrane associated loops, and more conclusively identified the amino acids in each TM-spanning region (Figure 1). The only region of the protein that has had its structure defined has been TMIV. Based on high-resolution nuclear magnetic resonance (NMR) analysis this essential TM-spanning segment is structured, but not as a traditional α -helix. TMIV is predicted to start with a series of β -turns, followed by an extended region, and end as a helix (126).

Further analysis of the membrane domain has also identified it as being the region responsible for NHE1 quaternary structure. NHE1 forms isoform specific homodimers *in vivo*, although the individual dimer subunits do function independently of each other (35).

Circular dichroism (CD) has been a useful tool in preliminary analysis of the C-terminal tail structure. The C-terminal 315 amino acids of human NHE1 were expressed in *E. coli* and purified. They were found to be ~35% α -helix, ~16% β -turn, and 49% random coil (42). Based on CD analysis of different protein fragments, the membrane proximal region was predicted as more compact and structured, possibly aiding in interaction with the membrane. The more distal region was flexible and unstructured, possibly allowing for structural changes associated with phosphorylation and protein interactions (42, 72).

The complete structure of the Na^+/H^+ exchanger isoform 1 has not been solved by high-resolution crystallography or NMR analysis, but the structure of the *E. coli* Na^+/H^+ antiporter NhaA has recently been published. Hunte *et al* describes NhaA as having a unique fold consisting of two dense transmembrane domains that show weak sequence homology but prove to be related structurally. The domains form periplasmic and cytoplasmic funnels that are not continuous in their structures, but do point towards each other. They suggest that a conformational change may open one tunnel to the other highlighting a possible mechanism (52). The eukaryotic Na^+/H^+ exchangers share little homology to the

prokaryotic Na^+/H^+ antiporters, but they do both exchange sodium for protons and are regulated by pH, so it is possible that essential features of NhaA structure and function also apply to mammalian NHE1.

3.2. Post-translational modifications

Sequence analysis of NHE1 predicts two potential glycosylation sites in the N-terminal domain (119). Subsequently, carbohydrate digestion and site-directed mutagenesis confirmed the existence and location of these modifications (26, 49). An N-linked glycosylation site was found at Asn 75 in the first extracellular loop in the N-terminal domain, in addition to O-linked glycosylation sites. When separated by SDS-PAGE two forms of NHE1 are consistently observed: a cell surface, 110 kDa protein that contains N- and O-linked oligosaccharides, and an 85 kDa protein that is likely intracellular and contains only the N-linked high-mannose oligosaccharide. Although the carbohydrate moieties exist *in vivo* they are not required for ion transport, nor are they necessary for proper folding and targeting of the protein (49).

An additional post-translational modification that occurs on NHE1 is phosphorylation in the last ~100 residues of the C-terminal tail. Initial reports of NHE1 being a phospho-protein also identified NHE1 phosphorylation as a regulatory mechanism (117). (Discussed in section 1.4.4)

4. Activity and Regulation of the Na^+/H^+ exchanger

4.1. Transcriptional regulation

The first level of protein regulation is gene transcription and translation, both of which affect the amount of protein produced and ultimately the collective activity of the protein pool. A region 1.1 kB upstream of the NHE1 gene transcriptional start site contains promoter and enhancer elements including an activator protein 2 (AP-2) binding site that is essential for NHE1 gene activity (33, 34), a chicken ovalbumin upstream promoter transcription factor (COUP-TF) binding site that acts as an enhancer (36), and a thyroid hormone and thyroid hormone receptor ($\text{TR}\alpha$) binding site that is also in the enhancer region at a site near but distinct from the COUP-TF binding site (73). Although binding sites exist, AP-2 transcription and COUP-TF may not be critical at all stages of development. In late stage embryonic mice the factors are not required for NHE1 gene activity, and NHE1 transcription and protein production occur in a tissue-specific and time-dependent manner (111, 112). Additionally, a number of mitogens such as insulin, thrombin, and epidermal growth factor stimulate NHE1 promoter activity, up regulating NHE1 expression (13). Overall, this suggests that transcription factors have a very specific role and that other factors are also involved in NHE1 gene regulation and protein production.

NHE1 gene regulation and protein production during a myocardial infarct has also gathered some attention because of the critical role of NHE1 in the

myocardium. Initially it was shown that the level of NHE1 mRNA increased upon low flow ischemia in the rat myocardium (33). It was later shown that after a myocardial infarct protein levels of both angiotensin II (AngII) and the angiotensin receptors subtype 1 and 2 (AT₁/AT₂) are increased, and that AngII via the AT₁ receptor controls NHE1 mRNA levels and protein production (116). Additionally, the κ -opioid receptor, when stimulated, has been shown to increase NHE1 gene expression in the heart (39).

4.2. Specific residues in the transmembrane domain

Individual residues in the transmembrane domain have been shown to be specifically involved in ion affinity, transport, and inhibitor sensitivity. In TMIV of human NHE1 Phe¹⁶¹, Phe¹⁶², Leu¹⁶³, and Gly¹⁷⁴ are critical for Na⁺ transport and inhibitor binding (24, 25, 137), additionally Phe¹⁶¹ has been shown to be a pore-lining residue (126). Site-directed mutagenesis studies have also shown that Pro¹⁶⁷ and Pro¹⁶⁸ are required for normal NHE activity, and when Pro¹⁶⁷ is mutated NHE1 expression and targeting is affected (125). In TMVII and extracellular loop 5 (EL5) Glu²⁶², Asp²⁶⁷, and Glu³⁹¹, three acidic residues, are critical for NHE activity and are possibly involved in sodium coordination (85). Residues Glu³⁴⁶ and Gly³⁵² in human NHE1 TMIX are also determinants of inhibitor sensitivity and cation translocation, and interestingly the interaction with Glu³⁴⁶ appears to be specific for side chain size rather than charge (58, 87).

Additionally, there are important residues in TMXI and the adjacent intracellular loop 5 (IL5), but they differ from the previously mentioned TM residues in that they are not involved in cation transport or inhibitor interaction. Rather, residues Arg⁴⁴⁰, Gly⁴⁵⁵, and Gly⁴⁵⁶ are involved in H⁺-sensing (141, 142).

The phenomenon of H⁺-sensing involves the interaction of proton(s) at a site distinct from the proton transport site of NHE1 that results in a pH_i dependence for Na⁺/H⁺ exchange (4, 97). Decreasing pH_i results in an increased rate of Na⁺/H⁺ exchange without affecting extracellular Na⁺ or H⁺ affinity, or inhibitor sensitivity. Mutation of Arg⁴⁴⁰ in IL5 or Gly⁴⁵⁵ and Gly⁴⁵⁶ in TMXI shifts the pH_i dependence of Na⁺ uptake to more acidic or alkaline respectively, indicating these residues are important for proper functioning of the H⁺-sensor.

4.3. Binding proteins

The C-terminal tail of the NHE1 has been shown to comprise the regulatory domain of the Na^+/H^+ exchanger (140). The regulatory role the cytoplasmic domain plays is largely due to its involvement with a number of binding proteins (Figure 1) that act to alter the configuration of the tail and its association with the membrane domain, as well as acting to convey cellular messages to the exchanger.

Phosphatidylinositol 4, 5-bisphosphate (PIP₂)

PIP₂ is a signaling phospholipid in eukaryotic plasma membranes. PIP₂ can also be converted to a number of other signaling molecules including inositol 1,4,5-triphosphate and (IP₃) and diacylglycerol (28), both of which are important 2nd messengers. The level of cellular PIP₂ is directly affected by the amount of available ATP since PIP₂ concentrations are dynamic and dependent on the activity of kinases and phosphatases (131). It has been well documented that NHE1, although not directly consuming ATP, is affected by cellular ATP depletion (44). There are several pieces of evidence that suggest that the Na^+/H^+ exchanger's ATP-dependence is due to an association with PIP₂, the first being PIP₂ plasmalemmal reduction being concurrent with ATP depletion (1). Additionally, NHE1 has two potential PIP₂ binding sites, amino acids 513-520 and 556-564, and mutation of these sites reduces the Na^+/H^+ exchange capability.

Finally, PIP₂ can bind the C-terminal tail of NHE1 *in vitro*(1). On the whole PIP₂ is an essential factor in pH_i regulation by NHE1 although it is yet to be determined *in vivo* whether this is via a direct or indirect interaction.

Calcineurin homologous protein 1 (CHP1)

CHP1 was originally identified as a NHE1 binding protein by screening an expression library with the C-terminal domain of NHE1 (74). It was later determined that CHP1 interacted with a hydrophobic cluster of residues between amino acids 510-530 of the NHE1 cytoplasmic domain, a region proximal to the membrane domain (92). The association between NHE1 and CHP1 has been proposed to be an essential interaction, as NHE1 or CHP1 mutants that impede binding result in a dramatic loss of Na⁺/H⁺ exchange activity. Characterization of the CHP1-NHE1 association suggests that it is a Ca²⁺-dependent, high affinity interaction that occurs at the plasma membrane (94). CHP1 possess four EF-hand motifs, which are very common calcium binding motifs, although it appears as though only EF3 and EF4 actually coordinate Ca²⁺. Mutation of either EF3 or EF4 reduces NHE1 pH_i sensitivity, and mutation of both results in poor interaction with NHE1. Additionally, NHE1 levels dictate the amount of endogenous CHP1 produced, and appear to be the main factor in recruitment of the protein to the plasma membrane (94).

CHP2, an isoform that has 61% amino acid identity to CHP1, has also been shown to interact with NHE1 (93). This protein is expressed at high levels in tumor cells and the association of CHP2 with NHE1 protects cells from serum deprivation-induced death by increasing pH_i . It is proposed that CHP2-NHE1 association maintains the malignant state of transformed cells (93).

Ezrin, radixin, and moesin (ERM) protein family

The cytoskeleton of a cell is essential for maintaining cell shape and establishing the mechanical and chemical properties of the plasma membrane. ERM proteins form important links between the actin filaments that form the structural basis of the cytoskeleton, and integral proteins that are situated within the plasma membrane (138). The NHE1 C-terminal tail contains two ERM protein binding motifs of acidic residues within amino acids 553-564, that mediate a direct interaction between the proteins (31). This interaction is pivotal in determining the role that NHE1 plays in a number of important cellular events such as cell migration, formation of signaling complexes, and resistance to apoptosis (30, 31, 151). When NHE1 and ERM physically associate the pro-survival kinase, Akt, is activated thus apoptosis is stalled. Additionally, association of NHE1 with ERM directs NHE1 localization in the lamellapodia of migrating cells. If this interaction is disrupted NHE1 is not properly localized and cells develop an irregular shape and poor motility.

Calmodulin (CaM)

Calcium is an important 2nd messenger in the cell that communicates its message by associating with proteins and altering their functional state. CaM is a Ca^{2+} binding protein that plays a major role in regulating NHE1 function in response to Ca^{2+} signaling. CaM binds Ca^{2+} and then binds to NHE1 at two sites in the cytoplasmic tail: residues 636-656 constitute a high affinity site, and residues 657-700 a low affinity site (12). The high affinity CaM binding site appears to interact with another region of NHE1 in the absence of CaM and Ca^{2+} in an autoinhibitory manner. Residues Leu⁶³⁹, Lys⁶⁵¹, and Tyr⁶⁵² are particularly important in the interaction (143). When CaM and Ca^{2+} are present they bind to the high affinity site thereby blocking the autoinhibitory interaction and activating the Na^+/H^+ exchanger (12). Additionally, there are seven conserved acidic amino acids, ⁷⁵³EEDEDD⁷⁵⁹, in the distal region of the C-terminal tail that play a role in CaM binding (70). CaM does not directly bind to this region of the tail but mutation of the sequence results in decreased NHE1 activity following an acid load and reduced CaM binding. It is possible that these residues are important for maintaining a proper conformation of the cytosolic tail that is required for CaM binding.

Carbonic Anhydrase II (CAII)

CAII is an enzyme that catalyzes the production of HCO_3^- and H^+ from the hydration of CO_2 . CAII associates with NHE1 *in vivo* via interaction at residues 790-802 of the C-terminal tail with Ser⁷⁹⁶ and Asp⁷⁹⁷ forming part of the binding site (71). Association of these two proteins increases NHE1 activity, and is dependent on the phosphorylation state of the NHE1 (69). It appears as though a region upstream of the CAII binding site, when dephosphorylated, interferes with CAII binding (71). The association of CAII with NHE1 may result in more efficient removal of proton's that are produced by CAII activity.

Tescalcin

Tescalcin is a Ca^{2+} binding protein that is structurally homologous to CHP. The protein has been shown to interact with the C-terminal tail of NHE1 *in vivo* in a Ca^{2+} dependent manner at a site distinct from CaM (72, 76). This association has an inhibitory effect on NHE1 activity.

4.4. NHE1 phosphorylation and kinases

Phosphorylation was identified as an NHE1 regulatory mechanism when Sardet *et al* confirmed that the protein was phosphorylated in response to treatment with thrombin (127), epidermal growth factor (EGF), phorbol esters, or serum, in a time-course concurrent with increased NHE1 activity (117). The importance of phosphorylation was further reinforced when tryptic mapping of the exchanger identified that a common pattern of phosphorylation and pH_i alkalization occurred after treatment of cells with Tb and okadaic acid, a Ser/Thr protein phosphatase 1 and 2A (PP1 and PP2A) inhibitor (118). The results from these studies suggested that some unknown kinase(s), activated by growth factors, phosphorylated the NHE1 on serine and/or threonine residues to mediate Na⁺/H⁺ exchange activation. Using deletion analysis Wakabayashi *et al* localized the major sites of *in vivo* phosphorylation to the residues 636-815 of the cytoplasmic tail, and confirmed the importance of this regulatory mechanism when they found that when the region was removed, the exchanger lost 50% of the stimulatory effect induced by growth factors (139). It has been postulated that the mechanism by which phosphorylation alters the activation state of NHE1 is by inducing a conformational change in the cytoplasmic tail and thereby altering the association of the tail with the H⁺ sensor of the NHE transmembrane domain (140).

Since identifying NHE1 as a phospho-protein there has been considerable interest in isolating the kinases responsible as well as the pathways connecting the

extracellular signals to the final event. Since phorbol esters were identified as a stimulator of NHE1 activity and inducer of NHE1 phosphorylation (117) the possibility of protein kinase C (PKC) being a NHE1 kinase was investigated. *In vitro* assays confirmed that PKC did not phosphorylate the last 178 residues of NHE1, nor did PKA, but Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) did. Analysis of the NHE1 amino acid sequence revealed three CaM kinase II consensus sequences, suggesting the possibility that phosphorylation by CaM kinase II may also occur *in vivo* (38). This was the first study that identified a kinase that directly acted on the Na^+/H^+ exchanger isoform 1.

The mitogen-activated protein kinases (MAPK) extracellular regulated kinases 1 and 2 (ERK1/2) have also been implicated in growth factor NHE1 activation. When inhibiting the ERK signaling cascade a 50-60% reduction in growth factor induced NHE1 stimulation was observed in Chinese hamster fibroblast cells, which agrees with the effect observed when removing the NHE1 phosphorylated region (15). Fast protein liquid chromatography (FPLC) isolated four fractions from rabbit skeletal muscle that were able to phosphorylate the NHE1 C-terminus, and when antibodies were used to immunoprecipitate MAPK from the extracts three of these four fractions lost this ability (146). This identified the three fractions as containing MAPKs involved in direct phosphorylation of NHE1 *in vitro*. Additionally, a downstream target of the ERK1/2 kinases, ribosomal protein S6 kinase (p90^{RSK}), was found to be activated

in vascular smooth muscle cells of rats after AngII treatment, which resulted in increased NHE1 activity (100, 132). It was later established that p90^{RSK} was a serum-stimulated kinase that directly phosphorylated NHE1 at Ser⁷⁰³ *in vivo* (133). This cemented the ERK1/2 kinases and their downstream target p90^{RSK} as being major players in growth factor-stimulated NHE1 activation. Further *in vitro* work also identified the residues Ser⁶⁹³, Ser⁷⁶⁶, Ser⁷⁷⁰, Thr⁷⁷⁹, and Ser⁷⁸⁵ as also being phosphorylated by ERK2, but the importance of these residues must be confirmed *in vivo* (75).

A number of other kinases have been implicated in NHE1 phosphorylation including p38, which is also a MAPK, p160ROCK, and the Nck-interacting kinase (NIK). Evidence for p38 in NHE1 phosphorylation agrees that the kinase does indeed phosphorylate the protein, but the effect on the Na⁺/H⁺ exchange is inconsistent. AngII treatment of rat vascular smooth muscle cells results in rapid activation of p38, resulting in NHE1 inhibition while providing a stimulatory signal to the exchanger via activation of ERK1/2 (62). In contrast the opposite effect was found in studying cytokine withdrawal in mouse B-cells. In this case activation of NHE1 induced intracellular alkalinization and triggered apoptosis possibly through phosphorylation of NHE1 at Thr⁷¹⁸, Ser⁷²³, Ser⁷²⁶, and Ser⁷²⁹ (human sequence) (59). The difference in results is likely due to activation of different pathways in different cell types, suggesting a further level of complexity

to kinase and NHE1 activation whereby the same proteins can provide different effects depending on the cell type.

The kinase p160ROCK is a downstream target of the GTPase RhoA, which has been shown to stimulate NHE1 activity (51). RhoA activation of NHE1 is mediated by p160ROCK, likely via a direct phosphorylation of NHE1. When the phosphorylation region, amino acids 636-815 of NHE1, is removed the effect of p160ROCK is blocked (136). NIK also activates NHE1 via phosphorylation at a site distal to residue 638, but in order to mediate its effect it must also bind NHE1 at the residues 538-638 of the cytoplasmic tail (153).

For phosphorylation to be considered a plausible mode of regulation for NHE1 activity, a system must also be in place for removing the phosphate group from the protein. To date the only confirmed NHE1 phosphatase is protein phosphatase 1 (PPI) (83). PPI was found to bind to NHE1 *in vivo* and reduce its basal level of Na^+/H^+ exchange activity. Additionally, inhibition of PPI by expression of inhibitor 2 (I2) elevated NHE1 proton efflux rates. These results suggest that PPI is involved in NHE1 regulation through its role in dephosphorylation of the protein. Prevention of dephosphorylation has also been shown to maintain NHE1 in an active state. The ligand 14-3-3 binds NHE1 at Ser⁷⁰³ after the residue has been phosphorylated by p90^{RSK} as a result of serum-stimulation, and limits dephosphorylation (68).

5. Stimulation of Na^+/H^+ exchanger activity

Stimulation of the Na^+/H^+ exchanger isoform 1 is an intricate process that involves a variety of mechanisms and pathways, making it a complicated system.

5.1. Growth factors and hormones

As stated above, it was determined very early on that growth factors and hormones stimulate Na^+/H^+ exchanger activation, but it required much work to elucidate the mode by which this occurred. Various binding proteins and protein kinases have been identified that interact directly and indirectly with the exchanger, but identifying the numerous proteins that are downstream from the stimulatory pathways adds to the complexity of the research. Some of the more notable growth factors and hormones that have been shown to activate NHE1 include: thrombin (127), serum, epidermal growth factor (EGF), insulin, angiotensin II (AngII), and lysophosphatidic acid (LPA) (15, 100, 117, 129, 136).

Tb activates the NHE1 via phosphorylation dependent and independent mechanisms (117, 139). Tb is a serine protease that interacts with the protease-activated receptor-1 (PAR-1) with high affinity (115). Interaction of Tb with its receptor initiates a cascade that activates ERK1/2 via Raf-1 activation of MAP-ERK kinase 1 (MEK1) resulting in NHE1 phosphorylation and activation (16, 118). Additionally, in rat ventricular myocytes, Tb activates NHE1 by means of a

PKC-mediated mechanism, although PKC does not directly phosphorylate the exchanger (38, 154).

Activation of NHE1 by serum involves a similar pathway as Tb activation but an interesting feature of this mechanism has been identified. The ERK1/2 activated p90^{RSK} has been shown to phosphorylate NHE1 at Ser⁷⁰³ (133) as stated above, but in doing so it also forms a 14-3-3 ligand binding site (68). When 14-3-3 binds to the phospho-Ser⁷⁰³ it limits de-phosphorylation of this residue and as a result, participates in serum-mediated NHE1 activation. Like serum, EGF activation of NHE1 appears to be similar to that of Tb, and results in NHE1 phosphorylation (118). Though Maly *et al* challenge the involvement of the MAPK pathway in EGF-mediated NHE1 activation (78). Their study only implicated PKC α in EGF-mediated NHE1 activation in mouse NIH3T3 cells. They suggest that that lack of MAPK involvement may be unique to their cell system. The hormone insulin activates NHE1 via a ERK1/2 MAPK pathway in addition to a PKC pathway (15, 120). In human erythrocytes insulin activates phosphatidylinositol 3-kinase, which in turn activates PKC ζ , and ultimately activates NHE1 (120).

AngII is a potent vasoconstrictor that plays an important role in determining blood pressure as well as stimulating ion fluxes, protein phosphorylation, contractility, gene expression, and cell growth. It acts via its interaction with AT₁ receptor, a G-protein coupled receptor (GPCR) (121). In rat vascular smooth

muscle cells (VSMC), AngII stimulates NHE1-mediated pH_i alkalinization via p90^{RSK} direct phosphorylation of NHE1 (100, 132). The p90^{RSK} kinase is activated via a MEK1→ERK1/2 pathway and is Ca²⁺-dependent, although not PKC-dependent. AngII-mediated NHE1 activation is also dependent on p38 activation. p38 is activated by AngII in rat VSMC but it negatively regulates ERK1/2 and NHE1, so AngII signaling appears to activate opposing pathways, and therefore must stimulate NHE1 through a balance of these pathways (62).

LPA is an anionic, bioactive phospholipid that mediates its signaling by associating with the GPCRs, LPA receptor subtypes 1-4 (LPA₁₋₄) (61). Association with the LPA receptors results in a variety of cellular responses including survival and proliferation, migration, and tumor-cell invasion (40). LPA is able to activate the Na⁺/H⁺ exchanger isoform 1 by stimulation of G_{α13} and activation of both the RhoA and Cdc42 pathways (51). Activation of the RhoA pathway results in p160ROCK activation, leading to NHE1 stimulation possibly by direct phosphorylation (136). Activation of the Cdc42 pathway mediates ERK1/2 activation leading to NHE1 stimulation (51, 145).

5.2. Other stimulatory mechanisms

Sustained intracellular acidosis treatment of neonatal rat ventricular myocytes (NRVM) results in a significant increase in NHE1 activity (50). Sustained intracellular acidosis was achieved by subjecting the cells to an NH_4Cl induced acid load, and then washing out the NH_4Cl with Na^+ -free buffer. If the cells were incubated in the Na^+ -free buffer for a prolonged period of time the low pH_i was maintained. In order to rescue the cells from the acid load Na^+ was reintroduced. To confirm that this mode of stimulation was not unique to the NRVM the authors of this study also looked at the effect on adult ventricular myocytes and the monkey COS-7 non-myocyte cell line. They found that there were no differences from what they observed in the NRVM (50).

This method of stimulation involved incubation of the cells in a Na^+ -free buffer, which could result in the depletion of internal Na^+ . To eliminate the possibility that the increased NHE1 activity was due to the reintroduction of external Na^+ , the authors completed activity assays where sustained intracellular acidosis was achieved by the addition of the specific NHE1 inhibitor cariporide (50). The inhibitory effect of cariporide is reversible with its removal from the buffer. These assays, completed for the three cell types, produced the same results as those observed with the sustained acidosis mediated by the absence of Na^+ . It is therefore unlikely that Na^+_i is involved in this effect.

To investigate the role that kinases played in the sustained intracellular acidosis method of NHE1 stimulation, the authors tested the ability of NRVM cell extracts to phosphorylate an NHE1-GST fusion protein (50). The fusion protein contained the last 178 amino acids of the NHE1 C-terminal tail. They found that the cell extracts contained significant NHE1 kinase activity resulting in increased NHE1 phosphorylation. Further analysis demonstrated that this method of stimulation activated ERK1/2 and p90^{RSK} kinases with a timeline corresponding to NHE1 activation. Additionally, inhibition of the ERK1/2 upstream effector MEK1 with PD98059 or U0126, two MEK1/2 inhibitors, prevented the stimulatory effect of sustained intracellular acidosis. Based on the results of this study, sustained intracellular acidosis initiates a signaling cascade that activates ERK1/2 and p90^{RSK} kinases and promotes NHE1 phosphorylation, ultimately activating Na⁺/H⁺ exchange.

Cellular osmotic challenge also activates the Na⁺/H⁺ exchanger (46). Osmotic stimulation does activate a number of MAP kinases, although this occurs in parallel to NHE1 stimulation rather than being the mechanism of NHE1 osmotic activation, which occurs through a phosphorylation-independent mechanism (43). The site that appears to mediate the osmotic stimulation of NHE1 exists between the end of the membrane associated domain and residue 566 of the tail (14). This region of the protein is involved in associations with the cellular cytoskeletal network, suggesting the possibility that shrinkage associated with cellular osmotic

stress may affect the NHE1 association with the cytoskeleton and therefore alter some yet elucidated regulatory mechanism (31).

Other mechanisms that stimulate NHE1 include H_2O_2 in rat cardiomyocytes (149), and more recently, zinc (32) in colonocytes (8). H_2O_2 causes activation of ERK1/2 kinases, in addition to causing an increase in NHE1 phosphorylation and activation, which may contribute to contractile dysfunction during ischemia-reperfusion injury (128). The mechanism by which Zn activates NHE1 occurs with the initial binding of Zn to an extracellular zinc-sensing receptor, which triggers the release of Ca^{2+} that subsequently activates ERK1/2 via the IP_3 pathway.

6. The Na^+/H^+ exchanger in health and disease

6.1. Physiological roles

A number of studies have used knock out or inhibition of NHE1 to determine its physiological role. Early studies examined the role of NHE1 in Chinese hamster lung fibroblasts cells (CCL39) by examining cell growth of cells deficient in NHE1 (103). In the absence of Na^+/H^+ exchange activity, cells lacked the ability to grow at neutral or acidic pH. To examine the role of NHE1 in intact mice an NHE1 knockout mouse was made. The *Nhe1*^{-/-} mice experienced a decreased rate of postnatal growth and exhibited ataxia and epileptic-like seizures (11, 27). The role NHE1 plays in cell cycle progression may be the basis for its involvement in cell growth and proliferation. An NHE1-dependent transient increase in pH_i promotes the G2/M transition allowing for S phase to occur in a timely manner (106). In the absence of Na^+/H^+ exchange the onset of S phase is delayed and mitosis is stalled. Additionally, NHE1 has been shown to regulate the expression of a number of genes involved in cell cycle progression via its control of pH_i (105).

NHE1 is also involved in cell differentiation. Treatment of cells with an NHE1 inhibitor prevents retinoic acid-induced differentiation (147). The involvement of NHE1 in cell growth, proliferation, and differentiation implicates the protein as being an important player in normal developmental processes.

Anchoring of NHE1 to the cytoskeleton *via* interactions with the ERM family

of proteins also links NHE1 to a role in maintenance of cytoskeletal structure, focal adhesion, and cell migration. NHE1-deficient cells have impaired cell adhesion, and cells that express NHE1 but that are unable to interact with the ERM proteins have reduced actin stress fiber organization, irregular cell shape, and impaired migration (30, 31, 135).

NHE1 is also thought to be involved in apoptosis, although its role seems to differ depending on the cells tested. In mouse B-cells during trophic factor withdrawal, which triggers pH_i dysregulation and apoptosis, NHE1 is activated leading to cellular alkalinization and progression of apoptosis (59). In contrast, NHE1 has also been shown to activate the pro-survival kinase, Akt, in response to apoptotic stress, and in breast cancer cells and leukemic cells inhibition of NHE1 induces apoptosis, although since these are cancer cells the signaling pathway may not represent a typical mechanism in healthy cells (109, 110, 151).

6.2. Heart disease

The Na^+/H^+ exchanger isoform 1 has been identified as the cardiac specific NHE as it is the predominant isoform present on the cardiomyocyte sarcolemma (37). NHE1 was found to be responsible for approximately 50% of proton efflux in isolated perfused ferret hearts, and as such it is considered to play a critical role in maintaining pH_i homeostasis and contractility (45). Although NHE1 has a

major role in normal cardiac function it has also been implicated as contributing to several pathological states.

During ischemia and reperfusion in the myocardium Na^+/H^+ exchange activity contributes to overall cell damage making it a valuable target for pharmacological intervention. The role NHE1 plays in this damage is via its involvement in what is termed the "coupled exchanger theory" (2, 67). During ischemia anaerobic glycolysis occurs resulting in the production of protons, which serve to activate the Na^+/H^+ exchanger isoform 1. Activated NHE1 exchanges the H^+_{i} for Na^+_{e} leading to a rapid accumulation of sodium in the cell. The high sodium concentration drives the increase in Ca^{2+} via reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). The ultimate result of this process is that the buildup of Ca^{2+} in the cells triggers various pathways leading to cell death. A huge body of evidence suggests that inhibition of NHE1 during ischemia and reperfusion protects the myocardium from calcium overload. In various animal models NHE1 inhibition by drugs such as cariporide, amiloride, and EMD 85131 have proven to be cardioprotective (47, 54, 122).

However clinical trials have not been as promising. The GUARD During Ischemia Against Necrosis (GUARDIAN) study, which covered a wide range of clinical situations with a total of 11,590 patients, found that cariporide demonstrated no overall significant benefit (134). Any benefit observed was limited to the subset of patients who underwent coronary artery bypass graft

(CABG) surgery (7). These patients received cariporide prior to the onset of ischemia, and reperfusion occurred in a timely manner. Whereas, a much smaller trial (n=100 patients) found that cariporide had the potential to attenuate reperfusion injury when it was administered prior to reperfusion, thereby improving recovery after myocardial infarction (MI) (114).

The Evaluation of the Safety and Cardioprotective effects of eniporide in the Acute Myocardial Infarction (ESCAMI) study also looked at the effect of NHE1 inhibition on MI. Like the GUARDIAN study they too found that eniporide did not demonstrate any significant improvements on clinical outcome (157). The lack of benefit may have been due to the administration of the drug only during the reperfusion therapy (7). More recently, the Na⁺/H⁺ Exchange inhibition to Prevent coronary Events in acute cardiac conDITIONS (EXPEDITION) study, also looked at the effects of cariporide. It found that although it reduced MI due to CABG surgery induced ischemia, it increased mortality rates overall due to an increase in cerebrovascular side effects (57, 81). Based on the outcome of the animal model studies and clinical trials, it is clear that NHE1 is an important pharmacological target but that more research is necessary for success at the clinical level.

NHE1 activity has also been shown to be involved in the development of cardiac hypertrophy. Hypertrophy is the enlargement of an organ due to the growth of individual cells rather than an increase in the total number of cells.

Following myocardial injury the heart compensates for its loss of functional cells by initiating a hypertrophic response, but this can be a maladaptive response and is one of the component that underlies eventual heart failure (55).

A number of factors that initiate hypertrophic response have also been shown to be involved in activating Na^+/H^+ exchange activity or are dependent upon it. For example, altered mechanical load activates NHE1 and is thought to be an early step in hypertrophic initiation. When mechanical stress occurs in the presence of an NHE1 inhibitor, the hypertrophic signaling cascade is inhibited (152). Also, stimulation with endothelin-I (ET-I) or via the α_{1A} -adrenoreceptor (α_{1A} -AR) activates NHE1 activity by initiating a signaling cascade that results in ERK1/2 and p90^{RSK} activation, in addition to activating hypertrophy (6, 60, 129, 130). Finally, in several models it was shown directly that NHE1 inhibition prevents cardiac hypertrophy. This was demonstrated *in vivo* in rats subjected to MI (63, 155) and *in vitro* in isolated cardiomyocytes (56) Though in numerous animal models in which NHE1 inhibition has proven to be effective in preventing hypertrophy, it is still necessary to establish whether this form of therapy can be beneficial clinically (57).

6.3. Cancer

NHE1 is activated in response to a number of mitogenic factors and its activity mediates such processes as cell growth, proliferation, and migration (Section 1.6.1), aberrations of which are characteristic of malignant cells. A key feature of transformed cells is that they have an alkalinized pH_i relative to non-transformed cells, and it has been suggested that this disturbance in pH homeostasis corresponds to an increasing cancerous state (48). In fact, it has been demonstrated that NHE1 is involved in the altered pH_i of malignant cells, and that NHE1-dependent alkalization plays a pivotal role in the development of a transformed phenotype, while inhibition of the exchanger prevents it (80, 108, 110). In addition to establishing an alkaline cellular state, NHE1 has also been implicated as a key player in breast cancer cell invasion.

In healthy cells serum stimulates NHE1 activity, but in breast cancer cells a conflicting situation occurs where serum-deprivation activates NHE1 to induce cell motility and invasion (107). Cytoskeletal changes that result in the formation of pseudopodia in invasive tumor cells is NHE1-dependent and is prevented via NHE1 inhibition (65). The signaling cascade that initiates the NHE1-mediated cytoskeletal rearrangement involves RhoA phosphorylation and inhibition by PKA, which stops p160ROCK activation. This prevents p38-mediated inhibition of NHE1, ultimately resulting in NHE1 activation and subsequent invasiveness (22, 95).

In accordance with NHE1 being identified as having a role in tumorigenicity, it has garnered some attention as a possible treatment target. Inhibition of NHE1 prevented pH_i alkalization and resulted in an increase in apoptosis in leukemic cells with more than 90% of the cancer cells killed (110). Paclitaxel, a chemotherapy agent used for leukemia and lymphoma, has been shown to activate a pathway involving PKA and p38 inhibition of NHE1, also leading to cancer cell apoptosis (109). Interestingly, this study also showed that if NHE1 was specifically inhibited simultaneously with paclitaxel treatment, the amount of the drug required for apoptosis to be achieved was significantly reduced. These two examples highlight the role NHE1 plays in cancer cell tumorigenicity in addition to its potential in the future of cancer treatment.

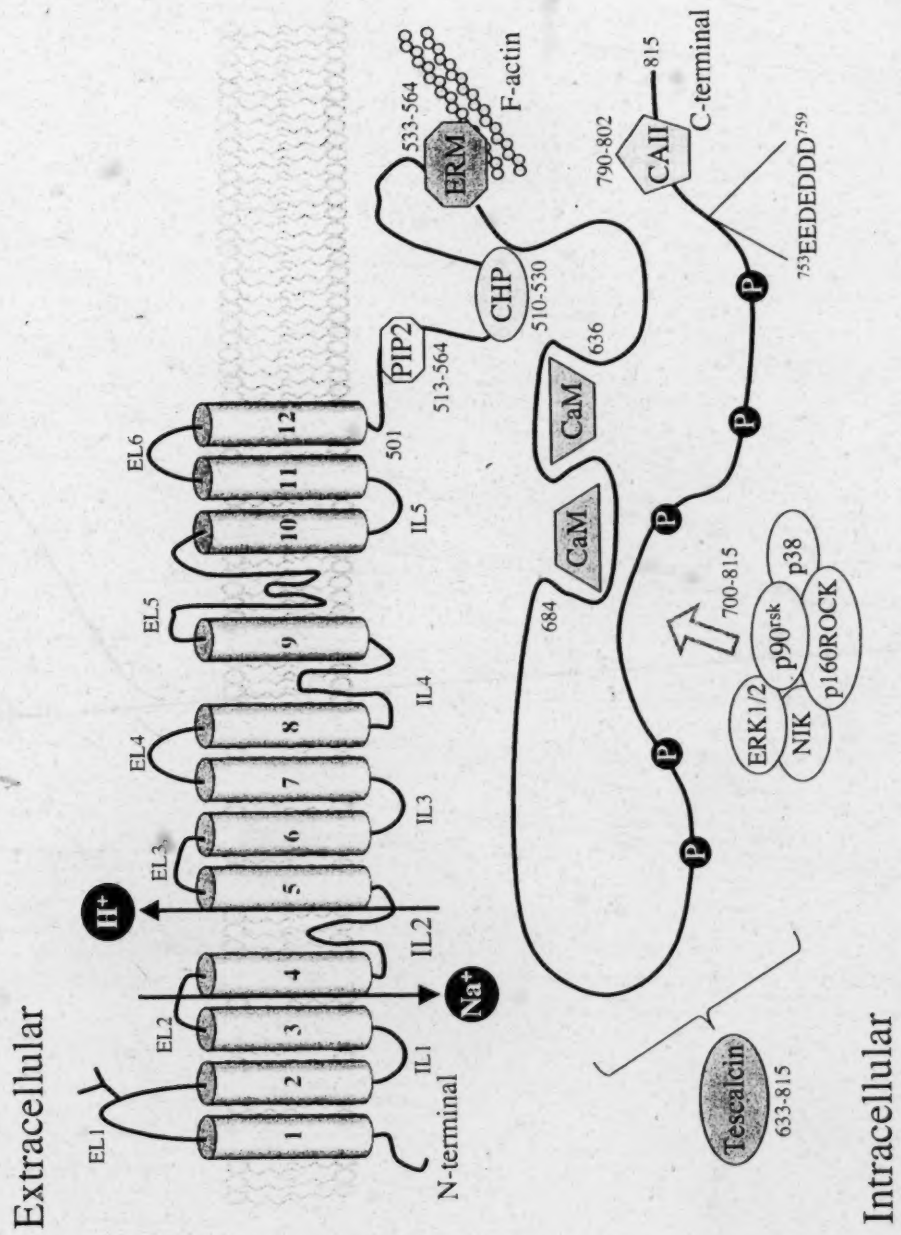
7. Thesis Objectives

The goal of this project was to further understand the role of phosphorylation in the stimulatory process of the Na^+/H^+ exchanger isoform 1 in intact cells. Specifically, we are interested in identifying what residues are involved in this process. Groups of serine and threonine residues in the phosphorylated region of the NHE1 cytoplasmic tail were mutated to alanine residues and the effect of mutation on NHE1 activation via a stimulatory pathway was assessed. The stimulatory pathway utilized was sustained intracellular acidosis treatment of cells which is an ERK-dependent pathway (50). We hope to demonstrate the importance of particular residues in the regulation of NHE1.

Figure 1. Putative topological diagram of NHE1 and regulatory elements.

EL1-EL6 Extracellular loop 1-6; IL1-IL5 Intracellular loop 1-5; PIP₂ Phosphatidylinositol bisphosphate; CHP Calcineurin homologous protein; ERM Ezrin/Radixin/Moesin proteins; CaM Calmodulin; CAII Carbonic anhydrase isoform 2; ERK1/2, p90^{rk}, NIK, p160ROCK, p38 Protein kinases; EEDEDDD Amino acids glutamic (E) & aspartic (D) acid (144).

D



Chapter II

Methods & Materials

1. Site-directed mutagenesis of NHE1

1.1. PCR

Polymerase chain reaction (PCR) was used to introduce DNA mutations in NHE1. The pYN4+ plasmid was used as the template (Figure 2). pYN4+ contains human NHE1 cDNA inserted downstream of a Rous Sarcoma Virus (RSV) promoter, an ampicillin resistance cassette (Amp^R), and a neomycin cassette. Primers were designed to introduce point mutations in the NHE1 cDNA that would result in translation of an alanine residue in replacement of either a serine or threonine residue. In addition to mutation for purposes of amino acid replacement, silent mutations were introduced to design new restriction enzyme digest sites. Forward and reverse primers used in the PCR reactions are listed in Table I.

The 50 µl PCR reactions contained: 250 µM dNTPs, 5 µl 10x PCR buffer (Roche), 1.5 mM MgCl₂, 0.5 µM forward primer, 0.5 µM reverse primer, 750 ng template DNA, 2.5 U PWO polymerase (Roche), and was made up to volume with ddH₂O.

The PCR protocol used is as follows:

1. 1 cycle of 95°C for 30"
2. 16 cycles of: 95°C, 30"; 55°C, 1'; 68°, 20'

PCR products were digested for 1hr at 37°C with 1 U Dpn I (New England Biolab) restriction enzyme to remove contaminating template DNA, and samples were run on 1% agarose gel to confirm PCR product.

1.2. Bacterial cell transformation

E. coli XL1-Blue strain was transformed by electroporation using the following parameters: 1300 V voltage, 25 mA current, 25 W power, 150 Ω resistance, 50 μ F capacitance. Transformed cells were transferred to 1 ml ice-cold LB media, and placed in a shaker for 45' at 37°C. Cells were then plated on LB agarose plates containing 100 μ g/ml ampicillin (Amp) and incubated at 37°C overnight.

1.3. Isolation of plasmid DNA from transformed bacteria

Single colonies of transformed *E. coli* were picked and cultured in 1.5 ml LB media containing 100 μ g/ml Amp and incubated overnight at 37°C in a shaker. Cells were harvested by centrifugation at 8,000g and plasmid DNA was isolated by the following miniprep protocol (refer to Table II for solution recipes).

Harvested cells were resuspended in 150 μ l of miniprep solution 1. 150 μ l of miniprep solution 2 was then added and allowed to sit for 5' at room temperature. Next, 150 μ l of miniprep solution 3 was added to the cell suspension and incubated on ice for 10'. The cell suspension was then centrifuged for 5' at

16,000g. The supernatant was transferred to a new tube and 150 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed by inverting the tubes, and centrifuged at 16,000g for 2'. The upper aqueous layer was transferred to a new tube, 1 ml 95% EtOH was added, and the solution was incubated at -70°C for 20'. To pellet the DNA the sample was centrifuged at 16,000g for 20'. The pellet was washed with 70% EtOH, and dried under vacuum. The dried pellet was resuspended in a pH 8.0 Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) containing 20 μ g/ml RNase for 30' at 37°C.

1.4. Restriction digestion of isolated plasmid DNA

To confirm that the PCR reaction had introduced mutations in the NHE1 cDNA restriction digests were completed to identify the presence of the newly introduced restriction site. The 20 μ l restriction digestion reactions contained: 5 μ l template DNA (isolated from single colonies of PCR transformed *E. coli*), 5-10 units of restriction enzyme, and 2 μ l 10x buffer (selected based on manufacturers recommendation, in the case of a double digest where two enzymes were used the buffer that best met the requirements of both enzymes was used). Table I illustrates the individual mutants and the enzymes used.

Restriction digest products were run on 1% agarose gels to confirm proper band patterns.

1.5. DNA sequencing

DNA sequencing confirmed the presence of proper DNA mutations. For DNA sequencing plasmid DNA was purified with a Qiagen® Maxiprep kit and resuspended in ddH₂O. Sequencing was completed by the University of Alberta, Department of Biochemistry, DNA Core Services lab.

2. Establishing mutant NHE1 proteins in mammalian cells

To test the mutations introduced in the NHE1 protein, stable cell lines were established in AP-1 cells. AP-1 cells are Chinese Hamster Ovary (CHO) cells that are deficient in all isoforms of plasma membrane NHE (113).

2.1. Transfection of AP-1 cells

Cells were plated on 100 mm dishes and allowed to grow to approximately 80-90% confluence in complete minimum essential medium (MEM) (SIGMA) media (refer to Table II for complete media contents) supplemented with 10% bovin growth serum (BGS). One day prior to transfection cells were washed with phosphate buffered saline (PBS) and complete α MEM supplemented with 0.5% BGS was added. The day of transfection the complete α MEM was replaced with complete α MEM without antibiotics and supplemented with 0.5% BGS. For each plate to be transfected the following complex was prepared. 24 μ g of plasmid DNA was diluted in 1.5 ml Opti-MEM® I Reduced Serum Medium (Invitrogen),

mixed gently by pipetting up and down, and incubated for 5' at room temperature. 60 μ l of Lipofectamine™ 2000 (Invitrogen) reagent was diluted in 1.5 ml Opti-MEM® I Reduced Serum Medium (Invitrogen), mixed gently by pipetting up and down, and incubated for 5' at room temperature. After the 5' incubations the diluted DNA was combined with the diluted Lipofectamine™ 2000 reagent, mixed gently by pipetting up and down, and incubated for 20' at room temperature to allow DNA-Lipofectamine™ 2000 complex formation. 3 ml of the DNA-Lipofectamine™ 2000 complexes were added to each 100mm dish of cells and mixed gently by rocking the plate back and forth. The cells were incubated at 37°C in 5% CO₂ for 4-6 hours at which time BGS was added to make a final concentration of 10%. Cells were further incubated at 37°C in 5% CO₂ overnight.

2.2. Establishing homogenous clones of stably transfected AP-1 cells

The day following transfection the media containing the DNA-Lipofectamine™ 2000 complexes was replaced with complete α MEM without antibiotics supplemented with 10% BGS and the cells were incubated at 37°C in 5% CO₂ overnight. The 2nd day following transfection the cells were passaged by trypsonization at 1:10, 1:100, 1:1000, and 1:10000 dilution to 100 mm dishes in complete α MEM supplemented with 10% BGS and 800 μ g/ml Geneticin® (G418) (Invitrogen). The media was changed every day for the next 3 days, and every 2nd

observed under the microscope at which point the concentration was then reduced to 600 $\mu\text{g/ml}$. When single colonies were visible to the naked eye G418 was reduced to 400 $\mu\text{g/ml}$. Single colonies were picked and transferred to a single well of a 12-well plate in 1.5 ml complete αMEM supplemented with 15% BGS.

Once cells in the 12-well plate were confluent they were passaged by trypsinization into two 60 mm dishes. One 60 mm dish was used to test for protein expression to confirm transfection, and the other was used to maintain the cell line.

2.3. Extraction of NHE1 protein from AP-1 cells

NHE1 protein was extracted from the plasma membrane using RIPA lysate buffer containing: 50 mM Tris (pH 7.4), 150 mM NaCl, 1% (v/v) NP-40, 0.25% (w/v) SDS, 0.1% (v/v) Triton X-100, 1 mM EGTA, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, and 0.1% (v/v) protease inhibitor cocktail (82)).

Confluent 60 mm dishes were placed on ice, washed with cold PBS, and 100 μl of the RIPA lysate buffer was added to each dish. The dishes were incubated on ice for 3' and the cells were then scraped off the dish with a disposable scraper for 1'. Using a pipet the cell lysate was collected and transferred to a 1.5 ml eppendorf tube and centrifuged for 5' at 16,000g at 4°C. The supernatant

containing the solubilized NHE1 protein was collected and the pellet containing various cell debris was discarded.

2.4. Western blot analysis of NHE1 expression

The RIPA lysate supernatant containing the solubilized NHE1 protein was mixed 3:1 with 4x SDS-PAGE loading dye and separated on a 10% acrylamide SDS-PAGE gel at 120 V. The SDS-PAGE gel was then transferred to a nitrocellulose membrane (Biorad) for 1 hour at 350 mA.

For reaction with antibodies the membrane was blocked by gently rocking in tris-buffered saline (TBS) with 10% w/v non-fat milk for 1 hour at room temperature. The blocked membrane was blotted with 1:2000 dilution of 12CA5 primary antibody (monoclonal mouse anti-HA antibody) in TBS with 1% w/v non-fat milk for 2 hours at room temperature. Next, the membrane was washed in TBS 4x for 15' at room temperature before labeling with 1:2000 dilution of goat-anti-mouse horse radish peroxidase (GAM-HRP) secondary antibody in TBS with 1% w/v non-fat milk for 1.5 hours at room temperature. Finally the membrane was washed in TBS 4x for 15' at room temperature and then developed using the Enhanced Chemiluminescence Assay (Amersham). The developed membrane was then used to expose X-ray film (FujiFilm). Exposed film was developed using a Kodak X-OMAT 2000 film processor.

3. Characterization of NHE1 mutants stably expressed in AP-1 cells

3.1. NHE1 protein expression

The BioRad DC Protein Assay kit was used to determine total protein content of RIPA lysates as per manufacturer's protocol. A volume of supernatant containing 50 µg of total protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted for HA-tagged NHE1. The Western blot was developed and used to expose film (FujiFilm). Exposed film was developed (Kodak X-OMAT 2000 Processor) and analyzed using the Image J (NIH, USA) densitometry program. The mutant NHE1 protein expression in AP-1 cells was compared to the level of expression of the wildtype NHE1 protein and was expressed as a percentage relative to wildtype.

3.2. NHE1 protein surface processing

Cells were plated and grown on 60 mm dishes in complete α MEM media supplemented with 10% BGS until approximately 80-90% confluent. The confluent cells were washed with PBS and then 4°C borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl_2 , 10 mM boric acid, pH 9.0). Cell surface proteins were labeled with biotin by incubating the cells in a 3 ml solution of membrane impermeable sulpho-NHS-SS-biotin (where NHS stands for *N*-hydroxysuccinimido; Pierce Chemical, Rockford, IL, U.S.A.) at 0.5 mg/ml in

borate buffer at 4°C for 30'. After the incubation the cells were washed three times with cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3), and then solubilized on ice in 500 µl of IP buffer (1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, HCl, pH 7.5, 0.1 mM benzamidine, 0.1 mM PMSF, 0.1% (v/v) protease inhibitor cocktail (82)). The cells were then scraped off the plate and transferred to a 1.5 ml eppendorf tube and centrifuged for 20' at 4°C at 16,000 g. The supernatant was collected and the pellet discarded.

200 µl of the supernatant was retained as the "total protein" for later SDS-PAGE analysis, while 200 µl of the remaining supernatant was incubated with 50 µl of immobilized streptavidin resin and incubated overnight at 4°C on a rocker to allow complex formation between streptavidin and biotin labeled proteins. The following day the incubated solution was centrifuged at 8,000 g for 2' to remove the streptavidin:biotin complexes and the supernatant was retained as the "unbound protein" fraction. The "total protein" and "unbound protein" fractions were separated on 10% acrylamide SDS-PAGE, transferred to a nitrocellulose membrane, and western blotted for the HA-tagged NHE1. By analyzing the relative amounts of "total protein" to "unbound protein" we could determine the amount of "bound protein" or biotin-labeled protein as a percentage of "total protein". The calculated "bound protein" indicates the amount of protein that is being properly processed and therefore reaching the cell surface.

3.3. NHE1 protein activity

Fluorometric activity assays were used to assess the ability of the NHE1 protein to recover the intracellular pH (pH_i) of a cell following an acid load. To study pH_i we can take advantage of the cell-impermeable, pH sensitive fluorescent dye, 2',7'-bis(carboxyethyl -5(and 6) -carboxy) fluorescein (BCECF). BCECF is available as an acetoxymethyl ester derivative (BCECF AM) that is cell permeable and non-fluorescent, but upon entering the cell the AM bond is cleaved by cellular esterases, releasing the BCECF (91).

Wildtype NHE1 transfected in AP-1 cells and each of the NHE1 mutants in AP-1 cells were plated in 35 mm dishes containing 2 glass coverslips in complete α MEM supplemented with 10% BGS. Cells were grown to approximately 80-90% confluence before the coverslip was removed and incubated in α MEM media supplemented with 0.5% BGS and 2 $\mu\text{g/ml}$ BCECF-AM for approximately 30'. After loading with the pH sensitive dye activity assays were completed on a PTI Fluorometer. Excitation wavelengths were 452 and 503 nm and the emission wavelength was 524 nm. The ratio of BCECF emissions at 524 gives a measurement of pH_i independent of dye concentrations (84).

The activity assay began by placing the BCECF loaded coverslip in a cuvette holder, and into a cuvette containing 2.5 ml of normal Na^+ buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgSO_4 , 5.5 mM glucose, 10 mM

HEPES, pH 7.3) at 37°C, and allowing the pH_i to stabilize for approximately 30 seconds. NH_4Cl was then added to the normal Na^+ buffer to a final concentration of 50 mM and incubated at 37°C for 3' to initiate an acid load. The coverslip was then removed from the NH_4Cl containing buffer and placed in a Na^+ -free buffer (135 mM N-methyl glucamine, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgSO_4 , 5.5 mM glucose, 10 mM HEPES, pH 7.3) for approximately 30 seconds, or until the pH_i had reached a minimum, at which point the coverslip was removed and placed in a normal Na^+ buffer (135 mM NaCl) and allowed to recover for 3'. Following the pH_i recovery a three-point pH calibration using Na^+ -free calibration buffers (135 mM N-methyl glucamine, 135 mM KCl, 1.8 mM CaCl_2 , 1 mM MgSO_4 , 5.5 mM glucose, 10 mM HEPES, at pH 6, 7, and 8) was completed, and was used to convert the fluorescence measurements to pH measurements.

The NHE1 protein activity was determined by measuring the slope of the first linear 20 seconds of the recovery period and was expressed as $\Delta\text{pH}/\text{sec}$.

4. NHE1 stimulation

4.1. Sustained acidosis

In order to stimulate the NHE1 in mammalian cells sustained intracellular acidosis was utilized (50). For all experiments that involved stimulation of the NHE1, one day prior to completing the experiment the cells were incubated at 37°C in 5% CO₂ in complete α MEM media supplemented with 0.5% BGS. The day of the experiment the following steps were completed for the control cells and stimulated cells.

Control:

1. Incubate cells in Na⁺ normal buffer at 37°C for 0.5-1'
2. Introduce 50 mM NH₄Cl to the Na⁺ normal buffer and incubate at 37°C for 3'
3. Remove NH₄Cl containing buffer and replace with Na⁺ normal buffer and incubate at 37°C for 3'

Stimulated:

1. Incubate cells in Na⁺ normal buffer at 37°C for 0.5-1'
2. Introduce 50 mM NH₄Cl to the Na⁺ normal buffer and incubate at 37°C for 3'

3. Remove NH_4Cl containing buffer and replace with Na^+ free buffer and incubate at 37°C for 3'
4. Replace Na^+ free buffer with Na^+ normal buffer and incubate at 37°C for 3'

4.2. 2-pulse NHE1 control and stimulated activity assays

As described in Section 2.3.3 the activity of NHE1 in mammalian cells was measured by a fluorometric activity assay. We used a 2-pulse assay to measure the effect of treatment on the activity of NHE1, while normalizing for the activity within various stable cell lines (Figure 3). The 2-pulse assay involves treating the cells plated on a coverslip as described for a regular activity assay up to the point of the 3-point pH calibration. Instead of beginning the calibration, cells are acidified with a second 50 mM NH_4Cl 3' incubation, followed by pH_i recovery in the Na^+ normal buffer. The second NH_4Cl pulse was either with or without a 3' sustained intracellular acidosis period. Following the pH_i recovery in Na^+ normal buffer a 3-point pH calibration was completed.

To calculate the effect of the stimulatory treatment the rate of recovery for the first pulse was set at 100% and the rate of recovery of the second pulse was expressed as a percentage relative to the first pulse (Figure 3). Using the percent values of the second pulse we compared the effect of sustained acidosis treated cells to control treated cells.

4.3. ERK1/2 & p90^{RSK} kinase activation

AP-1 cells stably expressing wildtype or mutant NHE1 were plated on 100 mm dishes and were treated as described in section 4.1 of the Materials & Methods. Following control or acidosis stimulatory treatment the cells were washed with cold PBS and 1 ml of cell lysis buffer (Table II) was added and the dishes were placed on dry ice for 5'. Cells were then allowed to thaw on ice for 15' before they were scraped off the dishes and transferred to chilled eppendorf tubes. The cell lysate was then sonicated for 10 seconds at constant duty with an output of 30 (Branson Sonifier). Next the cell lysate was centrifuged at 16,000g for 30' at 4°C. The supernatant was retained and the pellet discarded.

To analyze the kinases in the cell lysates the samples were run on 10% acrylamide SDS-PAGE, transferred to nitrocellulose membrane where proteins were identified by Western blotting.

4.4. ERK1/2 & p90^{RSK} Western blotting

Antibodies specific for the ERK1/2 and p90^{RSK} kinases were used to determine how much of the protein was loaded and transferred to the membrane. The antibodies were then stripped from the membrane by gently rocking the membrane at room temperature in a 0.2 M glycine solution at pH 2.8 for 10', then washing with both ddH₂O and TBS for 10' each. The membrane was then used in a second Western blot with antibodies specific for the phosphorylated kinases.

The second blots were used to identify the amount of protein that was activated in control and stimulated samples.

For ERK1/2, pERK1/2, and p90^{RSK} the Western blots were completed by gently rocking the membrane in TBS with 10% w/v non-fat milk for 1 hour at room temperature in order to block it. The blocked membrane was blotted with a 1:2000 dilution of primary antibody (see Table III for kinase specific antibodies) in TBS with 1% w/v non-fat milk for 2 hours at room temperature. The membrane was then washed in TBS 4x for 15' at room temperature before labeling with a 1:2000 dilution of secondary antibody (see Table III for kinases specific antibodies) in TBS with 1% (w/v) non-fat milk for 1.5 hours at room temperature. Finally, the membrane was washed in TBS 4x for 15' at room temperature before development with ECL (Amersham).

For Western blot analysis of phospho-p90^{RSK} kinase the membrane was blocked for 1 hour at room temperature in TBS-T (1 x TBS, 0.1% (v/v) Tween-20) and 5% w/v non-fat milk. The membrane was then washed three times for 5' in TBS-T before incubating in a TBS-T and 5% (w/v) bovine serum albumin (BSA) solution containing the 1:2000 dilution of primary antibody overnight at 4°C. The following day the membrane was washed three times for 5' in TBS-T and then labeled with the 1:2000 dilution of secondary antibody in a TBS-T and 5% (w/v) non-fat milk solution for 1.5 hours. Finally, the membrane was washed three times for 5' and developed using ECL (Amersham).

4.5. Inhibition of ERK1/2 & p90^{RSK} activation

The MEK1/2 kinase inhibitor U0126 (Sigma) at a concentration of 10 μ M in dimethylsulfoxide (DMSO) was used to inhibit activation of the ERK1/2 and p90^{RSK} kinases. The kinases were inhibited by incubating the plated cells in normal Na⁺ buffer containing the inhibitor for 10' before carrying on with the control or stimulation treatment. In all subsequent steps 10 μ M U0126 in DMSO was present in the buffer. As a control, similar experiments were completed with an equal volume of DMSO to confirm that the effects observed were due to the inhibitor and not due to the presence of DMSO.

4.6. *In vivo* phosphorylation of NHE1 protein

To examine *in vivo* phosphorylation of NHE1 proteins AP-1 cells were plated on 100 mm dishes. One day prior to experimentation cells were incubated in the complete α MEM supplemented with 0.5% BGS over night at 37°C in 5% CO₂. The day of experimentation the media was replaced with phosphate-free Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 0.5% BGS and incubated at 37°C in 5% CO₂ for 30'. The media was removed and replaced with 2 ml of the same media to which H₃³²PO₄ (Perkin Elmer) was added to a final concentration of 100 μ Ci/ml media. The cells were then incubated at 37°C in 5% CO₂ for 3 hours.

After the 3-hour loading period the cells were treated with either a control or acidosis stimulation. A cell lysate was prepared of a crudely purified collection of membrane proteins. Cells were lysed with 1 ml of detergent free RIPA buffer (150 mM NaCl, 80 mM NaF, 50 mM Tris-HCl at pH 8, 5 mM EDTA, 1 mM EGTA, 25 mM Na-pyrophosphate, 1 mM Na-orthovanadate, 0.1 mM benzamidine, 0.1 mM PMSF, 0.1% (v/v) protease inhibitor cocktail (82)). Freezing the cells on dry ice and thawing on ice three times augmented cell lysis. The lysed cells were scraped off the dish and transferred to polycarbonate tubes to be centrifuged at 35,000 rpm for 60' at 4°C. After centrifugation the supernatant was discarded and the membrane pellet was resuspended in 1 ml of detergent-containing RIPA buffer (150 mM NaCl, 80 mM NaF, 50 mM Tris-HCl at pH 8, 5 mM EDTA, 1 mM EGTA, 25 mM Na-pyrophosphate, 1 mM Na-orthovanadate, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 0.1 mM benzamidine, 0.1 mM PMSF, 0.1% (v/v) protease inhibitor cocktail (82)) and sonicated in a water bath sonicator (Bransonic 220) for 1'. The sonicated sample was centrifuged at 16,000 g for 30' at 4°C and the resulting supernatant was retained for further experimentation while the pellet was discarded.

4.7. Immunoprecipitation of NHE1 protein

The cell lysates prepared (described in section 2.4.6) were used for the immunoprecipitation (IP) of exogenously expressed NHE1 from AP-1 cells. Lysates for IP experiments were pre-cleared by rotating end-over-end at 4°C for 30' with 20 μ l of 100 mg/ml protein-A sepharose beads in detergent-containing RIPA buffer and 1% (w/v) BSA. To remove the beads the samples were centrifuged at 8,000 g for 2', and the supernatant was transferred to a clean eppendorf. The pre-cleared sample was rotated end-over-end with 1.4 μ g/ml of an anti-HA-antibody (Y-11, Santa Cruz Biotechnology Inc.) for 2 hours at 4°C. Meanwhile a 100 mg/ml solution of protein-A sepharose beads (Pierce) in detergent-containing RIPA buffer and 1% w/v BSA was rocking gently for at least 30' at 4°C to block the beads from any non-specific binding. After the blocking period the bead solution was centrifuged at 8,000 g for 2' and the supernatant removed. The sample bound with the HA-probe was added to the blocked protein-A sepharose beads and rotated end-over-end overnight at 4°C.

The following morning the samples were centrifuged to remove the supernatant. The beads were washed three times with 500 μ l of detergent-containing RIPA buffer to remove any unbound contaminating proteins. The bound protein was then eluted from the washed beads by incubating with 45 μ l of 1X SDS-PAGE sample loading buffer that contained 5% β -mercaptoethanol at 37°C for 15'. The tubes were then centrifuged at 8,000 g for 2', the sample was

collected and separated on 10% acrylamide SDS-PAGE, and then transferred to nitrocellulose membrane. The membrane was then blotted using the 12CA5 HA antibody to detect immunoprecipitated NHE1.

4.8. Analysis of NHE1 protein phosphorylation

The membranes containing the immunoprecipitated samples of control and stimulatory treated, *in vivo* phosphorylated NHE1 were used to expose film in order to identify any phosphorylated NHE1. We identified phosphorylated NHE1 by comparing the results from the autoradiographs to the corresponding Western blot using the 12CA5 antibody (anti-HA probe). Densitometry using Image J software was used to quantify both the levels of protein loaded and the amounts of phosphorylated protein, allowing for correction due to loading and transfer discrepancies. The effect of control and sustained acidosis stimulatory treatment was assessed simply by comparing the relative levels of phosphorylated protein between the two treatments and comparing the results of the mutants or inhibitory treated samples, to that of the wildtype NHE1 protein results. Comparisons of the various samples was achieved by expressing the phosphorylation levels as a ratio of phosphorylated NHE1 after stimulatory treatment over phosphorylated NHE1 after control treatment.

5. Statistics

For all experiments assessing the activity of the Na^+/H^+ exchanger the reported results were the product of four to twelve experiments. For all experiments where densitometry was utilized to analyze the data the reported results were the product of a minimum of three experiments. Statistical significance was determined using a Wilcoxon Mann-Whitney rank sum test.

Figure 2. pYN4+ plasmid

The pYN4+ plasmid was used for site-directed mutagenesis of NHE1 and mammalian cell transfection. The plasmid contains: Rous Sarcoma Virus long terminal repeat promoter (RSV-LTR(P)); Amp^R (ampicillin resistance gene); fl (+) ori (phage replication origin); ColE1 (bacterial replication origin); Neo (neomycin resistance cassette); PolyA (polyadenylation tail); Intron (upstream promoter and enhancer region of NHE1)

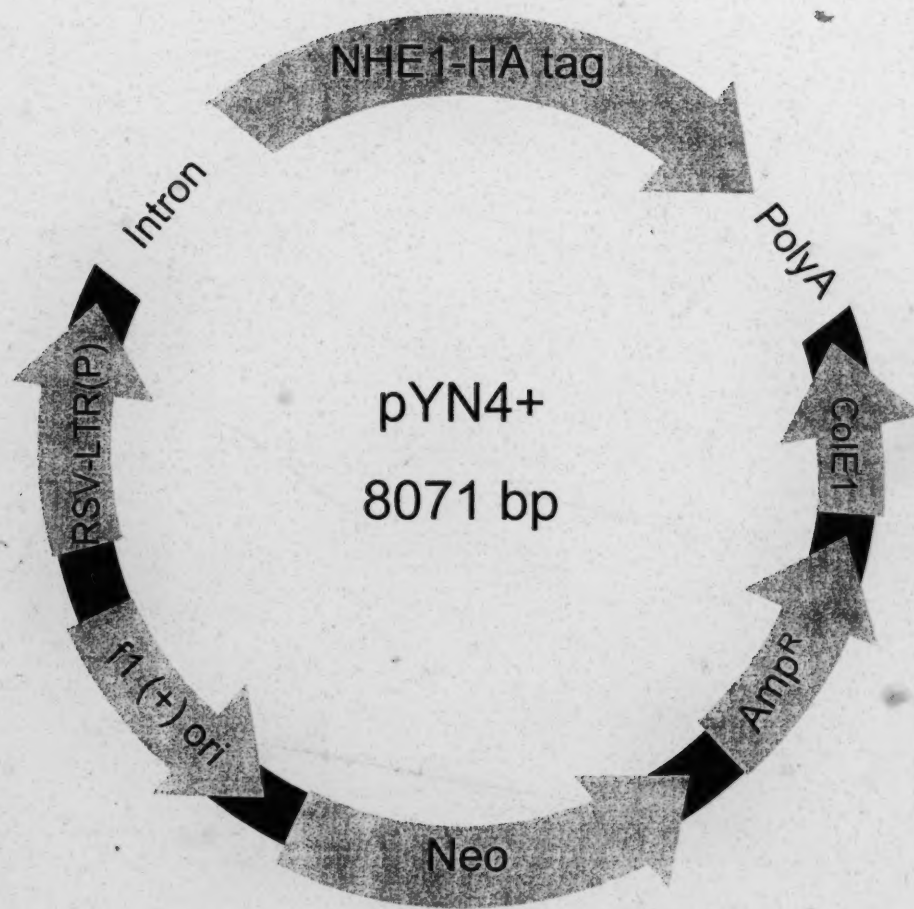
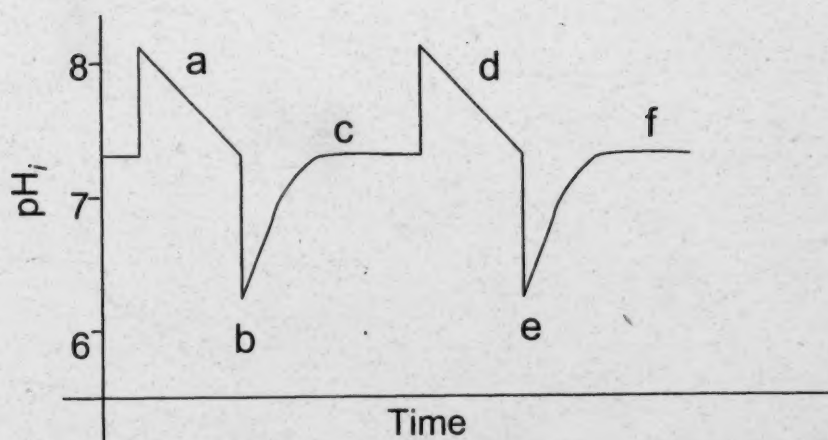


Figure 3. Control and sustained intracellular acidosis treatment procedure.

The upper graph depicts a control treated assay while the lower panel depicts a sustained intracellular acidosis treated assay. Both treatments begin in a Na^+ containing buffer. NH_4Cl is present in the regions a and d. The removal of NH_4Cl results in an acute acidification and a minimum pH_i is reached, labeled as b and e. In the case of the sustained acidosis treated cells, the acidification is allowed to progress for 3 min by incubating in a Na^+ -free buffer, this is labeled as g. Following acidification the cells are allowed to recover in a Na^+ containing buffer, this is labeled c and f. For further description of this treatment refer to the "Materials & Methods" section.

Control



Sustained Intracellular Acidosis

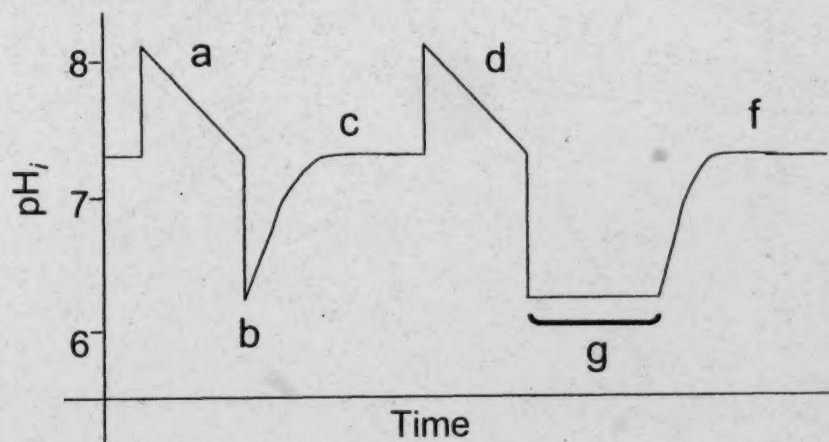


Table I: Primers designed for site-directed mutagenesis:

Round 1 and 2 refer to PCR reactions. Mutant T718A-S723/26/29A was made by completing two rounds of PCR. The first round (Round 1) introduced mutations S723/26/29A, while the second round (Round 2) introduced T718A.

Mutant	Primers	Restriction Site
S693A	F GCCCACAAAGCTTGACgCACCCACCATG R CATGGTGGGTGCGTcaAGCTTGTGGGC	Hind III
T718A-S723/726/729A	Round 1: F ATCGACCCCGGTgCCCCGCAGgCACCCGAGgCTGTaGAtCTGGTGAAT R ATTCACCAGaTctACAGcCTCGGGTgCTGCGGGGcAGCCGGGTCGAT Round 2: F AAGGAGGACCTaCCgGTCATcGCCATCGACCCCGGT R AGCCGGGTCGATGGCGATGACcGgtAGGTCCTCCTT	Round 1: Bgl I Round 2: Age I
S766/770/771A	F GCATCATGATGCGGgccaAGGAGACTgCagCCCCAGGAACCGAC R GTCGGTTCCTGGGGgtGcAGTCTCCTTGgCCCGCATCATGATGC	Pst I
T779A-S785A	F CCGACGATGTCTTCgCCCCCGCCcCgAGTGACgCCCCAGCTCCCGAG R CTGGGAGCTGGGGgcGTCACTCGGgCGGGGGcGAAGACATCGTCGG	Ava I

*Lowercase bases indicate mutations

*Underlined sections indicate newly introduced restriction sites

Table II: Solution recipes

Miniprep Solution 1	50 mM 25 mM 10 mM	Glucose TRIS (pH 8) EDTA
Miniprep Solution 2	0.2 M 1%	NaOH SDS
Miniprep Solution 3	3 M 1.5%	K-Acetate Glacial Acetic Acid
Complete α MEM	Media (SIGMA) HEPES L-Glutamine Gentamycin	500 ml 40 mM 0.25 mM 4 mg/ml
Cell lysis buffer	Na-Pyrophosphate NaF NaCl EDTA EGTA Na ₃ VO ₄ Tritol X-100 HEPES pH 7.4 PMSF Benzamidine Protease inhibitor Cocktail (82)	50 mM 50 mM 50 mM 5mM 5 mM 0.1 mM 0.1% (v/v) 10 mM 0.1 mM 0.1 mM 0.1% (v/v)
RIPA lysis buffer	Tris pH 7.4 NaCl NP-40 SDS Triton X-100 PMSF Benzamidine Protease inhibitor Cocktail (82)	50 mM 150 mM 1% (v/v) 0.25% (w/v) 0.1% (v/v) 0.1 mM 0.1 mM 0.1% (v/v)

Table III: ERK1/2 & p90^{RSK} Western blot antibodies

Protein	ERK1	ERK2	Phospho- ERK1/2	p90 ^{RSK}	Phospho- p90 ^{RSK}
1 ^o Antibody	Anti-ERK1 goat monoclonal SCB	Anti-ERK2 rabbit monoclonal SCB	Anti- pERK1/2 mouse monoclonal SCB	Anti- p90 ^{RSK} goat monoclonal SCB	Anti- Phospho- p90 ^{RSK} (Ser380) rabbit monoclonal CST
2 ^o Antibody	Rabbit anti- goat HRP polyclonal	Goat anti rabbit HRP polyclonal	Goat anti mouse HRP polyclonal	Rabbit anti goat HRP polyclonal	Goat anti rabbit HRP polyclonal

SCB – Santa Cruz Biotechnology

CST – Cell Signaling Technology

HRP – Horse radish peroxidase

Chapter III

Results

1. Establishing stable cell lines expressing NHE1 protein mutants

To further understand the regulatory mechanisms of NHE1 it is useful and convenient to establish mutant NHE1 protein expressing cell lines that can be used for a variety of experiments and provide *in vivo* results. By using mammalian cell lines expressing our protein we can assess how mutations can effect regulation of the protein in the complex system of cell signaling and protein-protein interactions. From this we can infer the importance of particular residues in functioning of the protein. While stable lines require more time to establish than transient transfection, they offer several benefits. These include a constant, lower level of expression than transient transfectants. This allows for healthier cells and more precise measurement of activities. Additionally, we are able to establish a homogeneous cell line from a single transfected cell. This prevents interference by untransfected cells. Finally, we are able to complete numerous experiments on the cell line without having to considering any variations due to transfection. Since the cell line is homogeneous the population of cells should remain unchanged experiment to experiment.

1.1. Site-directed mutagenesis of NHE1

To exchange specific serine and threonine residues within the wildtype NHE1 protein site-directed mutagenesis was used. Primers (Table I) were designed that introduced base-pair substitution in the NHE1 cDNA that resulted in Ser/Thr to alanine replacement at the protein level, and silent base-pair mutations introduced new restriction enzyme digestion sites at the DNA level. The template used was the pYN4+ plasmid (Figure 2), which contains the NHE1 coding region with a triple HA-tag at the C-terminus. A PCR reaction was carried out using the mutagenic primers, the pYN4+ template, and the proofreading enzyme, PWO polymerase (Roche). The PCR product was then used to transform bacterial cells and plasmid DNA was harvested from single colonies. DNA restriction digests were completed in order to identify mutated pYN4+ plasmid DNA and DNA sequencing confirmed proper and complete mutagenesis.

1.2. Transfection of AP-1 cells

Plasmid DNA containing specific site-directed mutations was used to transfect the mammalian AP-1 cell line. AP-1 cells are Chinese hamster ovary cells that are deficient in plasma membrane NHE protein, making them a useful tool in studying exogenous NHE1 protein without endogenous protein interference (113). To establish stable cell lines LipofectamineTM 2000 reagent was used, and the transfected cells were plated so as to give rise to colonies from a single transfected cell. This method allowed for establishment of a homogenous

collection of cells expressing the mutant protein. Transformation was confirmed by Western blotting using 12CA5 as the primary antibody.

2. Characterization of NHE1 protein mutants

As a control for all subsequent experiments AP-1 cells stably transformed with the pYN4+ plasmid were used. This cell line expresses the NHE1 protein that contains no mutations and is referred to through out this text as the 'wildtype' NHE1 protein.

For all cell lines expressing the mutant NHE1 proteins, at least 2 separate stably expressing clones were tested for protein expression and NHE1 activity. There were not substantial differences between the individual clones, and therefore a single clone was selected and used in all subsequent experiments.

2.1. Analysis of mutant protein expression

To assess the effects mutation had on expression of the transfected NHE1 proteins, RIPA lysates of the stably transfected AP-1 cell lines were prepared for the wildtype protein and each of the mutants, in addition to untransfected AP-1 cells. 50 µg of total protein was separated on SDS-PAGE and transferred to nitrocellulose for Western blotting with the 12CA5 antibody. Visualization of the Western blot by ECL (Amersham) showed the relative levels of NHE1 protein expressed, and can be seen in Figure 4a.

The densitometry program Image J was used to determine the mutant NHE1 expression levels in AP-1 cells relative to the wildtype NHE1 protein in AP-1 cells. If wildtype NHE1 expressed at 100% and untransfected AP-1 cells showed 0% expression of an HA-tagged NHE1 protein, the mutant expression was as follows: S⁶⁹³A 69 ± 4%, T⁷¹⁸A-S^{723/26/29}A 40 ± 10%, S^{766/70/71}A 118 ± 4%, T⁷⁷⁹A-S⁷⁸⁵A 68 ± 6% (Figure 4b). There was considerable variation in the expression levels, although all mutants produced a measurable level of NHE1 protein.

In addition to confirming protein expression this set of experiments also provides information of the effects mutation might have on maturation of the protein. NHE1 protein within the cell can be observed as two forms; a fully glycosylated 110 kDa protein that is believed to be the protein that reaches the plasma membrane, and a base glycosylated 85 kDa protein that is likely an immature version of the plasma membrane protein (26). The Western (Figure 4a) shows two distinct bands at ~110 kDa and ~85 kDa for the wildtype NHE1 protein and each of the mutants. Our laboratory has earlier observed that with protein miss-targeting, the NHE1 protein may be found predominantly as an 85 kDa base-glycosylated form (126). This was not the case with these mutant proteins.

2.2. Analysis of mutant protein localization

We examined NHE1 cell surface targeting to determine if the mutant proteins reached the plasma membrane similar to wildtype NHE1. Cell surface labeling and analysis of NHE1 targeting was as described in Section 2.3.2. By labeling the intact mammalian cells with biotin we are able to separate the biotin bound, surface labeled NHE1 from the biotin unbound, intracellular NHE1 protein. This separation allows for the distinction between the protein reaching the surface and that being retained within the cell. Figure 5a shows a Western blot of the total and unbound NHE1 protein from a surface processing experiment for the wildtype protein and each of the mutants expressed in AP-1 cells. A summary of the results for the amount of wildtype and mutant NHE1 protein reaching the cell surface for three separate experiments is shown in Figure 5b. The amount of NHE1 reaching the cell surface for the wildtype and mutants proteins ranges from 45% to 81%, and in no case is the protein being entirely retained within the cell. For wildtype NHE1 the level of surface protein was 69%, and the lowest level of expression was the S^{766/70/71}A mutant that was reduced only 24% below this level.

2.3. Analysis of mutant protein intracellular pH recovery

To assess the functional effects of mutagenesis on the Na⁺/H⁺ exchanger we measured the rate of pH_i recovery following an acute acid load and compared the mutants to the wildtype protein. Figure 6 shows the results for at least four

separate experiments of each cell type. All the mutant NHE1 proteins mediated a recovery following an acute acid load that was comparable to that of wildtype.

3. *In vivo* activation of NHE1 specific protein kinases

Our interest in the phosphorylated residues of the Na^+/H^+ exchanger is of particular importance during activation of the protein. Phosphorylation is a common method of post-translation modification used to regulate proteins, and has been implicated in NHE1 regulation earlier (117). The mechanism of activation used in this study was sustained intracellular acidosis, which acts through an ERK-dependent pathway (50). Initially we confirmed that the mechanism of activation of the wildtype Na^+/H^+ exchanger was present in AP-1 cells.

3.1. Sustained acidosis activation of NHE1

To test the effect of the sustained acidosis treatment on NHE1 activity we assessed the rate of pH_i recovery (RoR) following an acute acid load on the first pulse of a two-pulse assay. Cells were subjected to a second acid pulse whereby the acid loading was prolonged to 3 min before recovery was allowed (Figure 3). By comparing the RoR of the second pulse to that of the first we could observe any treatment related increases in activity. As a control, two-pulse assays were

completed where both pulses were acute acid loads, this allowed for correction of any assay related effects on NHE1 activity.

The results (Figure 7) show that for wildtype NHE1 protein in AP-1 cells, there is no significant difference between the rate of pH_i recovery of the first and second pulses of the control treated cells. The RoR of the 2nd pulse for each treatment are as follows: control 0.013 ± 0.02 ; stimulated 0.019 ± 0.02 (example trace Figure 7a). The stimulatory treatment resulted in a 1.5-fold increase in activity from the 1st to the 2nd pulse, which was statistically significant, while the control treatment did not alter the RoR. The results confirm that the sustained intracellular acidosis treatment has a stimulatory effect on the Na^+/H^+ exchanger in AP-1 cells.

3.2. ERK1/2 & p90^{RSK} activation by sustained acidosis

In addition to measuring the effect of sustained acidosis on NHE1 activation, we also assessed the effect the treatment has on activation of the kinases ERK1/2, p90^{RSK} . AP-1 cells expressing the wildtype NHE1 were subjected to either control or sustained acidosis stimulatory treatment, the cells were then lysed and the lysates was separated on SDS-PAGE and transferred to nitrocellulose membrane. Protein loading was measured by blotting the membranes with ~~the~~ antibodies specific for the proteins (Figure 8a & 8b). Protein activation was measured by

blotting the membrane for the phospho-proteins. The relative levels of protein and phospho-protein were determined using Image J densitometry software.

Figure 8c summarizes the results of three experiments. The results show that although ERK1 is not significantly activated, the ERK2 and p90^{RSK} kinases show a statistically significant increase in activation following the stimulatory treatment. ERK2 activation was 71% over the control values, whereas p90^{RSK} had a 56% increase in kinase activation relative to that of controls. ERK1 was activated by 24% but the increase was variable and not statistically significant.

3.3. Inhibition of ERK1/2 & p90^{RSK} activation

To link the activation of NHE1 specific kinases – ERK1/2 & p90^{RSK} – to NHE1 activation following sustained intracellular acidosis treatment, kinase inhibition experiments were completed. The MEK inhibitor U0126 was used to inhibit the MAPK pathway. MEK activates the ERK1/2 kinases and consequently p90^{RSK}. We examined the effects on NHE1 activity and phosphorylation level following an acute acid load in the presence of U0126.

To assess the effect on NHE1 activity four 2-pulse activity assay treatments were completed: a control 2-pulse assay plus DMSO; a stimulatory 2-pulse assay plus DMSO; a control 2-pulse assay plus 10 μ M U0126 in DMSO; and a stimulatory 2-pulse assay plus 10 μ M U0126 in DMSO. The rate of pH_i recovery (RoR) for the first and second pulse of each set of treatments was compared, with

the first pulse of each set of treatments set at 100% and the second pulse RoR expressed as a percentage relative to it. The results (Figure 9) show that in the absence of U0126 there is a statistically significant increase of 67% in NHE1 activity following sustained intracellular acidosis. Control cells did not show a difference in NHE1 activity between the first and second pulses in the presence of DMSO. In contrast, in the presence of 10 μ M U0126 (Figure 9) there was no longer an increase in exchanger activity following sustained intracellular acidosis. Both the control and stimulatory treatment in the presence of U0126 resulted in no significant increase in NHE1 activity during the second pulse RoR.

To assess the effect of MEK inhibition on NHE1 phosphorylation, four sets of experiments were completed: a control treatment; a stimulatory treatment; a control treatment plus 10 μ M U0126 in DMSO; a stimulatory treatment plus 10 μ M U0126 in DMSO. The phosphorylation levels of each treatment were corrected for loading and the level of phospho-NHE1 after positive stimulation was plotted (Figure 10). In the absence of the MEK inhibitor, U0126, the stimulated/control ratio of phospho-NHE1 was 1.7 ± 0.3 while in the presence of 10 μ M U0126 the ratio was 0.97 ± 0.08 . The phospho-NHE1 ratio in the presence of the inhibitor was significantly lower than that observed for phospho-NHE1 in the absence of the inhibitor.

4. *In vivo* stimulation of NHE1 protein mutants

To assess the effect of mutations of phosphorylation sites of the NHE1 cytoplasmic domain we examined NHE1 stimulatory activity and NHE1 phosphorylation levels following sustained intracellular acidosis.

4.1. Effect of C-terminal mutations on sustained acidosis activation of NHE1

Each mutant was subjected to either control or sustained acidosis treatment as described in section 3.3.1 and the rate of recovery following an acid load was compared. The results (Figure 11) demonstrate that the S⁶⁹³A and T⁷¹⁸A-S^{723/26/29}A mutants have a level of stimulation similar to that of the NHE1 wildtype protein. The T⁷⁷⁹A-S⁷⁸⁵A has lower level of stimulation however it still is not significantly different from wildtype. The graph also shows that the S^{766/70/71}A mutant does not have an increased level of activity and this is significantly different from that observed for the wildtype protein.

4.2. Analysis of NHE1 protein phosphorylation following control and sustained acidosis treatment

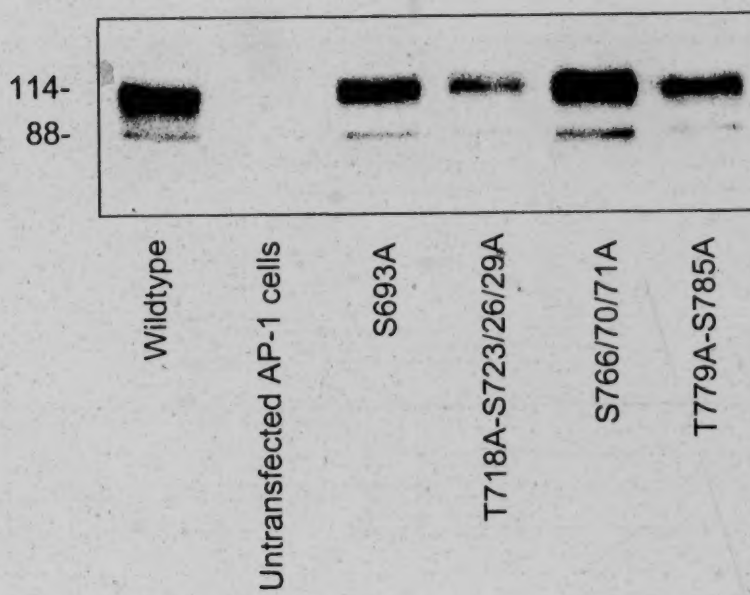
Since the NHE1 activity following sustained acidosis of the S^{766/70/71}A and T⁷⁷⁹A-S⁷⁸⁵A mutants both differed from that of wildtype NHE1, phosphorylation levels of the mutants were compared to that of wildtype. The stimulated/control phospho-NHE1 levels of the wildtype and the S^{766/70/71}A and T⁷⁷⁹A-S⁷⁸⁵A

mutations are as follows: 1.74 ± 0.32 , 0.75 ± 0.07 , and 1.28 ± 0.29 . Figure 12a illustrates a representative autoradiograph of the results while Figure 12b summarizes the results of at least six independent experiments. The S^{766/70/71}A mutation affected the level of NHE1 phosphorylation after sustained acidosis and the stimulated/control phospho-NHE1 ratio is significantly lower than that that observed for wildtype NHE1. The T⁷⁷⁹A-S⁷⁸⁵A mutation does appear to have a reduced effect on the level of phospho-NHE1 after sustained acidosis treatment relative to the wildtype protein but the difference was not statistically significant.

Figure 4. NHE1 protein expression.

A: Representative anti-HA antibody Western blot of untransfected AP-1 cells plus wildtype and mutant NHE1 expressed in AP-1 cells. 50 μ g of total protein was loaded for each sample. Molecular markers are given in kDa. B (next page): Summary of protein expression levels of wildtype and NHE1 mutants. Actual values are shown in the text below bars. The amount of NHE1 expressed was determined using Image J software, and the values were expressed relative to the amount of wildtype NHE1 expressed in AP-1 cells. The results are mean $\pm$ SE for 3 separate protein samples.

A



B

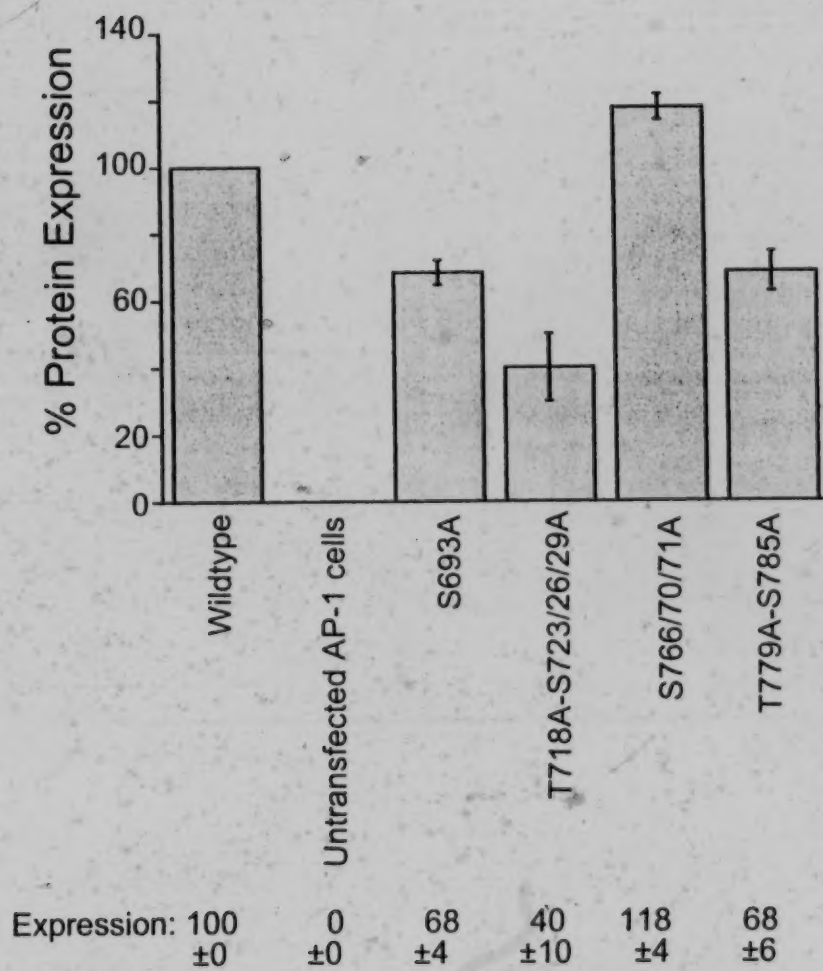
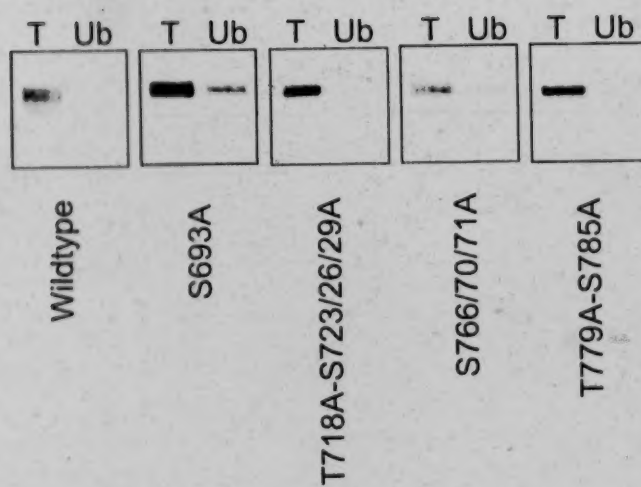


Figure 5. NHE1 protein localization.

A: Representative Western blots of wildtype and mutant NHE1 protein samples from surface processing experiments using anti-HA antibody. Equivalent amounts of total (T) and unbound (Ub) protein were used for each sample. B (next page): Summary of NHE1 protein levels in the plasma membrane. Actual values are in the text below. The amount of surface protein was calculated quantifying the difference between the T and Ub protein using Image J software. The values are expressed relative to total protein. The results are the mean $\pm$ SE of 3 separate experiments.

A



B

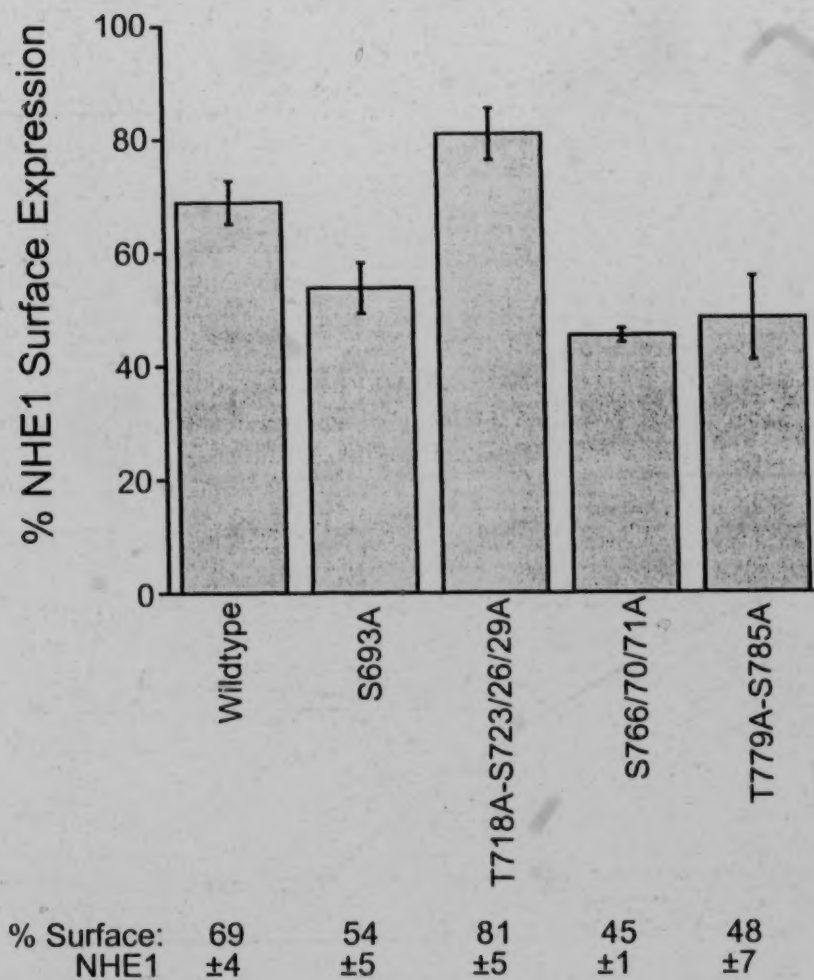


Figure 6. Rate of pH_i recovery from an acute acid load by AP-1 cells stably transfected with wildtype or mutant NHE1.

Values are expressed as $\Delta\text{pH}/\text{sec}$. The results are the mean $\pm$ SE of at least 4 separate assays.

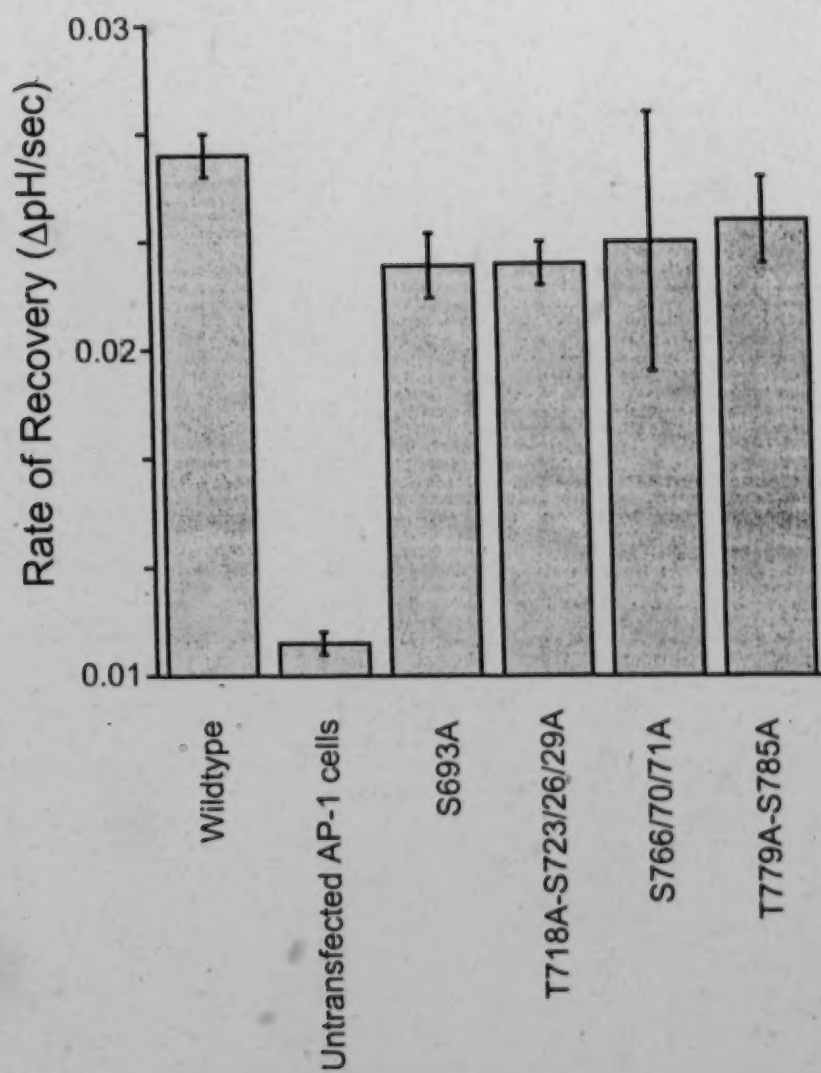


Figure 7. The effect of 3 minute sustained acidosis on wildtype NHE1 Activity

Dual ammonium chloride prepulse was used with or without sustained intracellular acidosis. A: Example trace of control and sustained intracellular acidosis stimulatory treatment. B: The rate of recovery for each acid pulse was determined and the values of the second pulse were compared to those of the first. "Control" indicates the second pulse was in the absence of sustained intracellular acidosis. "Stim" indicates the second pulse administered sustained acidosis. The results are the mean $\pm$ SE of at least 10 separate assays. $\star$ indicates that the 2nd pulse of the stimulated treatment is significantly higher than that of the control, at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

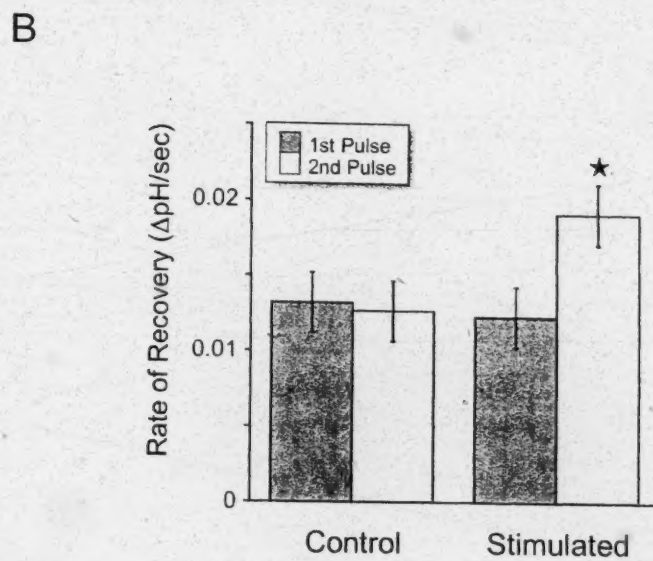
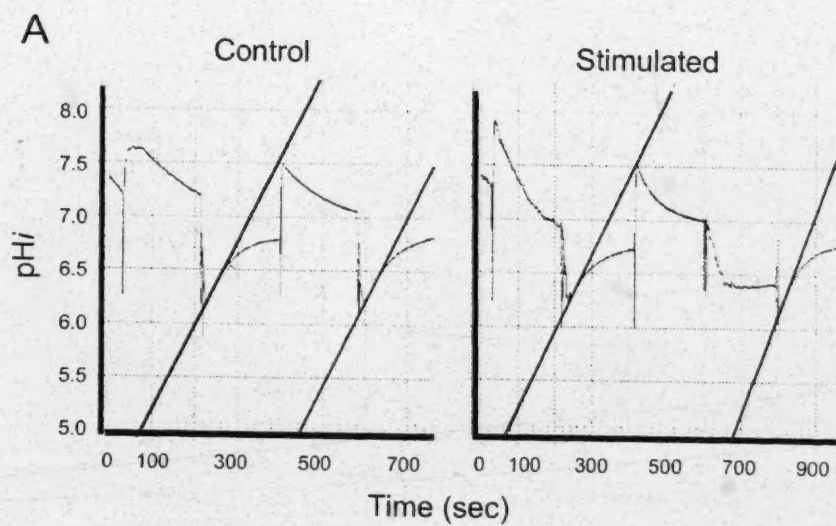
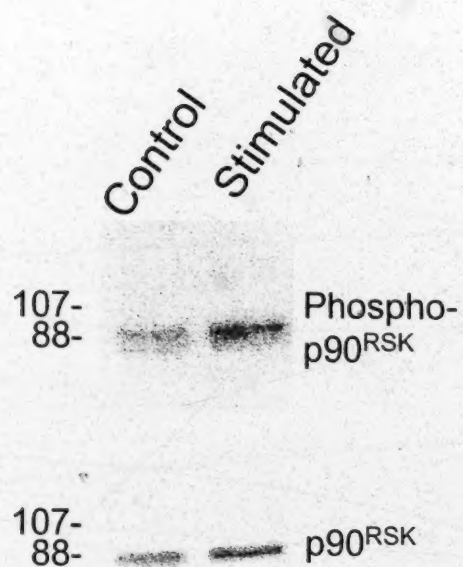


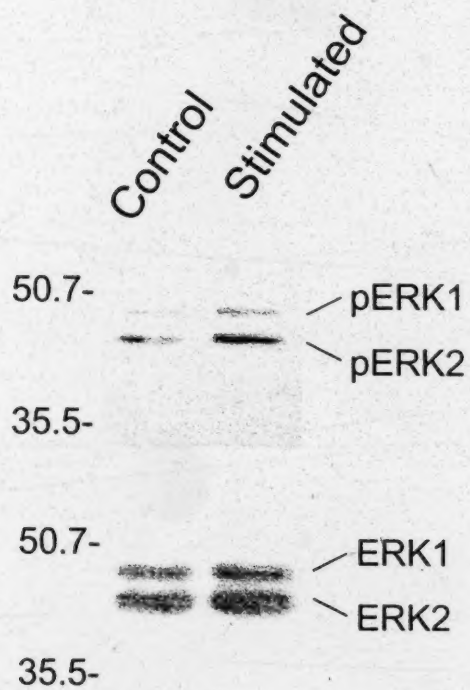
Figure 8. Effect of sustained acidosis on ERK1/2 and p90^{RSK} activation.

A. p90^{RSK} activation. Upper panel: Representative Western blot of p90^{RSK} from control or sustained acidosis treated cells blotted with an anti-phospho-p90^{RSK} antibody. Lower panel: Corresponding Western blot of p90^{RSK} blotted with anti-p90^{RSK} antibody. **B. ERK1/2 activation.** Upper panel: Representative Western blot of ERK1/2 from control or sustained acidosis treated cells blotted with an anti-phospho-ERK1/2 antibody. Lower panel: Corresponding western blot of ERK1/2 blotted with anti-ERK1 and anti-ERK2 antibodies. **C (next page).** Summary of kinase activation by sustained intracellular acidosis. The amounts of protein shown in A and B were quantified using Image J software and the amount of phospho-protein was corrected for protein loading. The level of activated kinase in the stimulated sample was expressed relative to the control sample. The results are the mean $\pm$ SE for 3 separate experiments. $\star$ indicates that the level of activated kinase in the stimulated treatment is significantly higher than that of the control treated at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

A



B



C

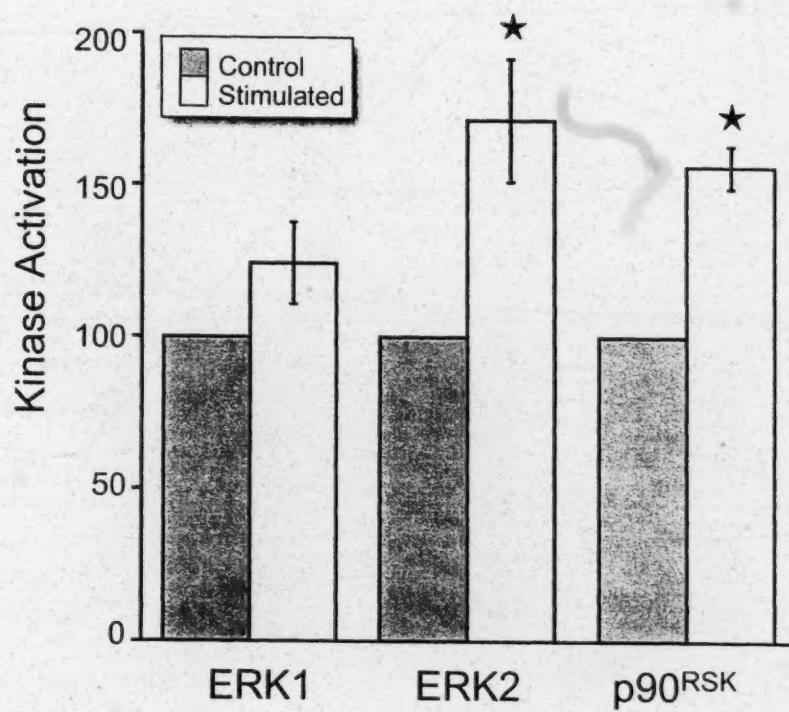


Figure 9. Effect of 10 μ M U0126 on sustained acidosis activation of NHE1.

Dual ammonium chloride prepulse was used with or without sustained intracellular acidosis. The rate of recovery for each acid pulse was determined and the values of the second pulse were compared to those of the first. "Control" indicates the second pulse was in the absence of sustained intracellular acidosis. "Stim" indicates the second pulse administered sustained acidosis. Additionally, these assays were completed in the presence or absence of the MEK1 inhibitor U0126 as described in the "Materials & Methods" section. The rate of recovery (RoR) for each pulse is plotted. The RoR values are expressed relative to the first pulse for each treatment. The results are the mean $\pm$ SE for at least 6 separate assays. $\star$ indicates that the 2nd pulse of the stimulated treatment is significantly higher than that of the control at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

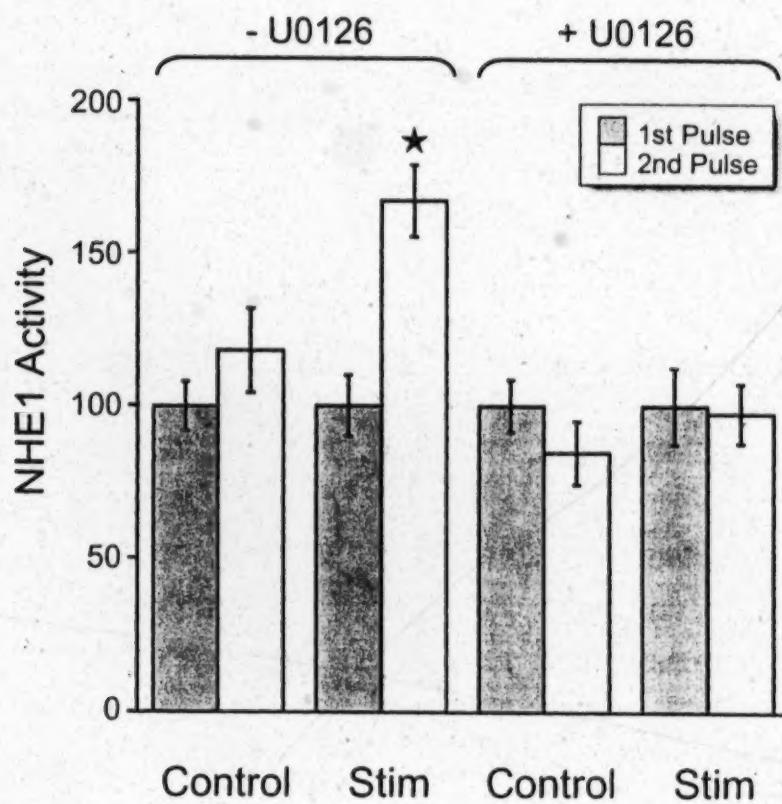


Figure 10. Effect of 10 μ M U0126 on sustained acidosis mediated NHE1 phosphorylation.

A: Cells were loaded with ^{32}P before being subjected to either control (C) or sustained acidosis (S) treatment in the presence or absence of 10 μ M U0126. A: Autoradiograph of immunoprecipitated NHE1. B (next page): Ratio of phosphorylated NHE1 from stimulated versus control cells. Phospho-NHE1 levels in autoradiographs were corrected for loading using values from anti-HA Westerns. The results are the mean $\pm$ SE for at least 5 separate experiments. $\star$ indicates that the stimulated/control ratio for phospho-NHE1 in the presence or absence of U0126 is significantly different at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

A

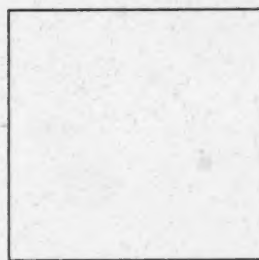
-U0126



C S

S/C ratio: 1.7 ± 0.3

+U0126



C S

0.97 ± 0.08

B

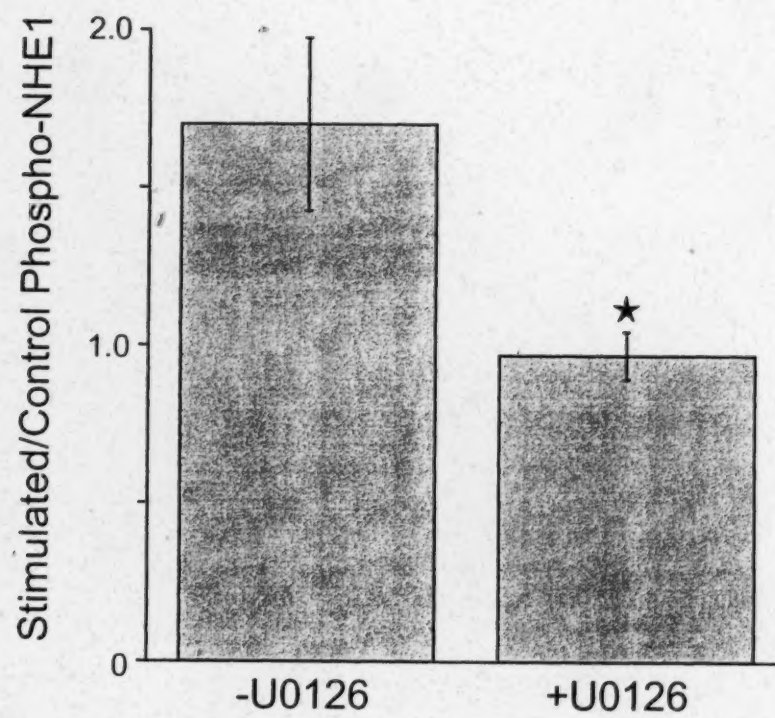


Figure 11. The effect of sustained acidosis on wildtype and mutant NHE1 rate of recovery.

Dual ammonium chloride prepulse was used with or without sustained intracellular acidosis. The rate of recovery for each acid pulse was determined and the values of the second pulse were compared to those of the first. "Control" indicates the second pulse was in the absence of sustained intracellular acidosis. "Stim" indicates the second pulse administered sustained acidosis. The results are the mean $\pm$ SE for at least 10 independent assays. $\star$ indicates that the mutant NHE1 activity after acidosis treatment, is significantly different from that observed in the wildtype NHE1 at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

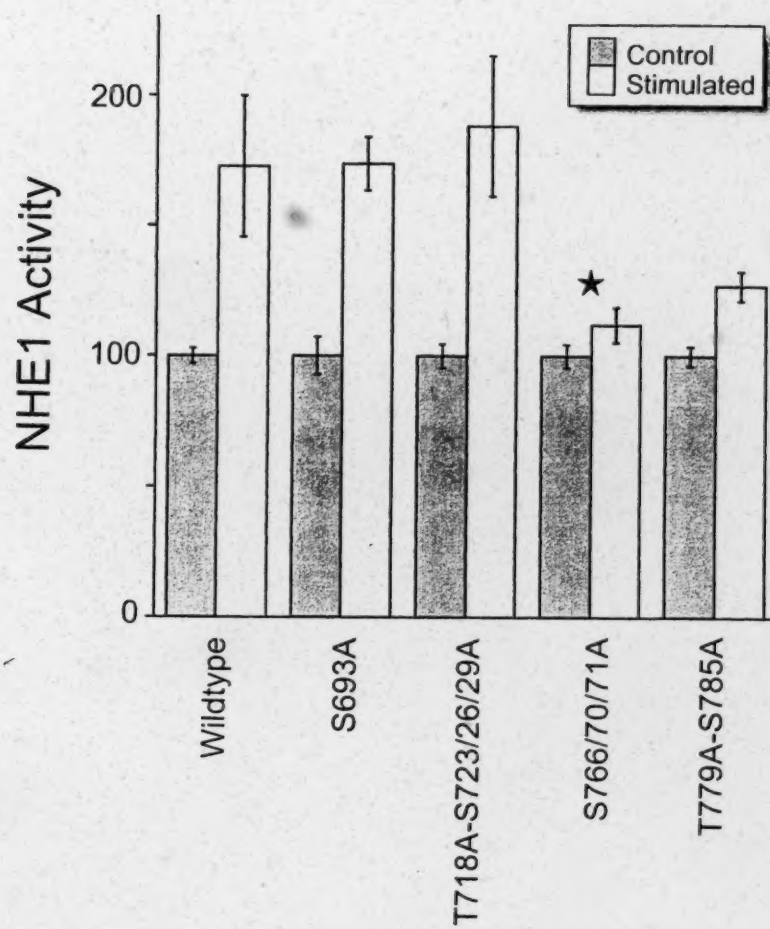
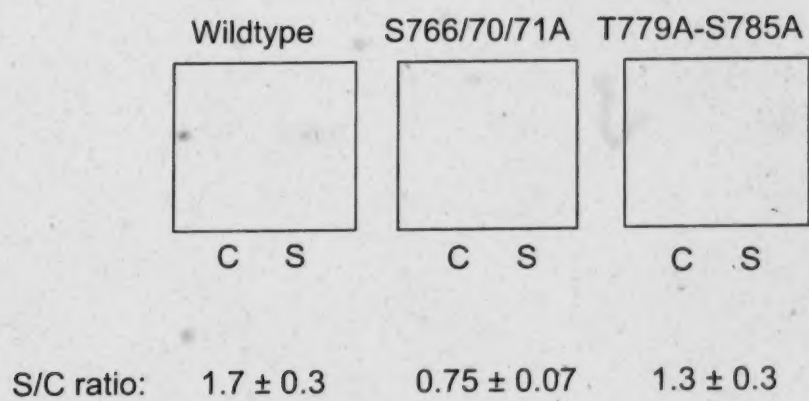


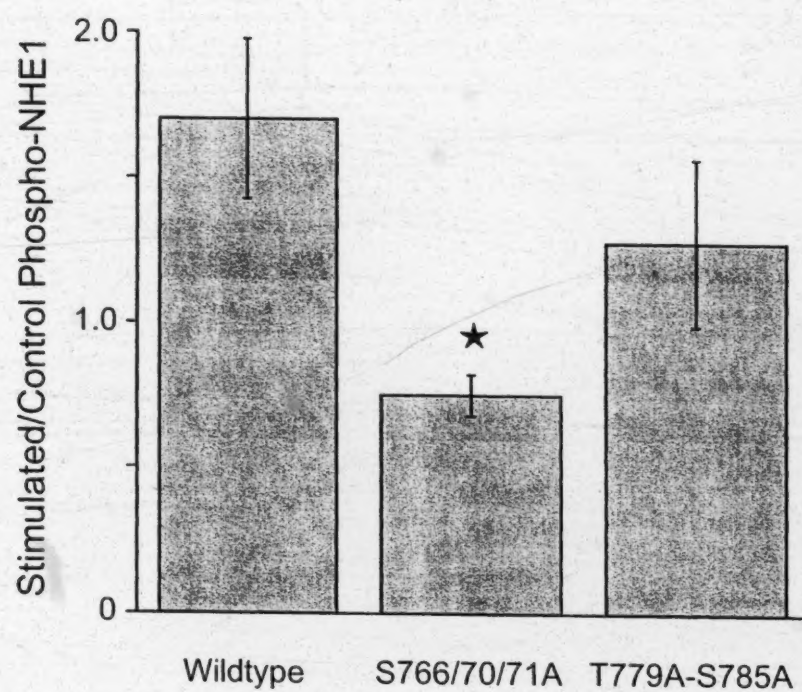
Figure 12. Sustained acidosis mediated NHE1 phosphorylation for wildtype and mutant NHE1.

A: Cells were loaded with ^{32}P before being subjected to either control (C) or sustained acidosis (S) treatment. A: Autoradiograph of immunoprecipitated NHE1. B (next page): Ratio of phosphorylated NHE1 from stimulated and control treated cells. Image J software was used to quantitate phospho-NHE1 levels in autoradiographs, and amounts were corrected for protein levels. The results are the mean $\pm$ SE of at least 6 separate experiments. $\star$ indicates that the stimulated/control ratio for phospho-NHE1 is significantly different than that observed for the wildtype protein at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test.

A



B



Chapter IV

Discussion

1. Discussion

1.1. Summary of NHE1 phosphorylation

As discussed in the introduction, phosphorylation is a valuable mechanism of reversible regulation for numerous proteins. It has the ability to act as a molecular switch whereby the addition or removal of a phosphate group can alter the functional state of a protein. For the Na^+/H^+ exchanger isoform I, phosphorylation mediated by various kinases is responsible for 50% of the stimulatory effect due to growth factors (139). Although several kinase consensus sites have been identified in the cytoplasmic domain, and we know that NHE1 is phosphorylated in response to growth factor stimulation, only one residue that gets phosphorylated *in vivo* has been conclusively identified (38, 117, 133). Residue Ser⁷⁰³ has been identified as a serum stimulated residue phosphorylated by p90^{RSK} kinase (133). This present study identifies a novel set of residues that play a critical role *in vivo* in NHE1 stimulation via sustained intracellular acidosis.

The C-terminal 180 amino acids of NHE1 have been implicated as being responsible for the stimulatory effect due to growth factors by our laboratory and others (84, 139, 146). To identify specific residues in the C-terminal tail involved in mediating NHE1 stimulation we designed several mutants. Our initial experiments showed that the mutant NHE1 proteins were expressed and properly targeted to the plasma membrane. Though the level of expression varied slightly,

all mutants were expressed and properly targeted such that their activity could be carefully analyzed.

1.2. Sustained intracellular acidosis mediated stimulation of NHE1

Sustained intracellular acidosis activates the Na^+/H^+ exchanger isoform I in cultured neonatal rat ventricular myocytes (NRVM) via activation of the ERK1/2 MAPK pathway (50). We initially confirmed that this same pathway was functional in AP-1 cells (Chinese hamster ovary cell line deficient in NHE) stably expressing human NHE1. A 3 minute sustained intracellular acidosis treatment of AP-1 cells expressing the human NHE1 protein resulted in 1.5-fold increase in activity (Figure 7). The increase in activity mediated by sustained intracellular acidosis is significant, but was not as large of an increase as observed by Haworth *et al* in the NRVM. There are several possible reasons for this discrepancy. First, Haworth *et al* looked at the activation of endogenous rat NHE1 in NRVM whereas we looked at human NHE1 stably expressed in adult CHO cells. The signaling pathway activated could have varying effects depending on the cell type, protein type, and developmental state the cell represents. For example, we have seen that NHE1 plays opposing roles in the apoptotic pathway depending on cell types studied, and our lab has shown that in mice NHE1 expression changes according to physiological role and developmental state of the animal (109, 110, 112, 151). Additionally there could be species-specific differences in the impact

this mode of stimulation has on the protein. Finally, the control activity of the NRVM is exceptionally low when compared to that observed in the AP-1 cells. It is possible that in the AP-1 cells there is already some basal level of stimulation of NHE1 that masks part of the effect of the sustained acidosis stimulation resulting in our observation of only a 1.5-fold increase in activation. Despite the difference from the report of Haworth *et al.*, the effect of acute acid load was significant and reproducible, allowing us to use it as a reliable method of NHE1 stimulation.

In order to assess the effect of sustained intracellular acidosis on kinase activation cells were subjected to either control or stimulatory treatment and the level of phosphorylated protein kinase was measured. The phosphorylated protein represents the activated forms of ERK1/2 and p90^{RSK} kinases. This experiment was completed in the presence of protease inhibitors to prevent protein degradation, and phosphatase inhibitors to prevent de-phosphorylation of activated kinases. Figure 8a and 8b show representative Western blots of the activated kinases and the corresponding blots of the total kinase present. Figure 8c depicts a summary of the results of 3 separate experiments and shows that both ERK2 and p90^{RSK} are activated as a result of the stimulatory treatment. Sustained intracellular acidosis resulted in a 75% increase in ERK2 activation and a 50% increase in p90^{RSK} activation. The activation of the kinases occurred with a time course consistent with that observed for sustained intracellular acidosis-mediated

stimulation of NHE1 activity. Once again the level of activation observed in our experiments were not as pronounced as that observed by Haworth *et al*, additionally we did not see the activation of ERK1 as a result of this treatment. The basis of the discrepancy is likely the same as that stated above. Also, as we observed for the activity assays, the control treated cells appeared to display a basal level of activated protein.

To draw a connection between the activation of ERK2 and p90^{RSK} kinases and the stimulation of NHE1 activity, MAPK inhibition experiments were completed. The MEK1 specific inhibitor, U0126, was used to block the activation of ERK1/2 and subsequently p90^{RSK} activation. At a concentration of 10 μ M, U0126 suppresses MEK1 activation by the Raf protein kinase, by ~50% without inhibiting other protein kinases (29). This makes it a useful drug for selective inhibition of the classical ERK1/2 mitogen activated protein kinase pathway.

Figure 9 illustrates that when sustained acidosis occurs in the absence of U0126 there is a significant increase in NHE1 activity (as discussed above). When sustained acidosis occurs in the presence of U0126 there is no stimulatory effect. From this observation we can conclude that sustained intracellular acidosis-mediated activation of NHE1 in AP-1 cells is the result of ERK2 and p90^{RSK} activation. Additionally we can see that this mechanism results in an increase in NHE1 phosphorylation *in vivo* (Figure 10) that is suppressed by prevention of ERK2 and p90^{RSK} kinase activation by U0126. Based on these

results we can imagine a pathway whereby sustained acidosis activates ERK2 and subsequently p90^{RSK} via the Raf→MEK1 cascade, leading to direct or indirect NHE1 phosphorylation.

An additional remark to make about the results shown in Figure 10 is that in the control treated cells NHE1 shows a basal level of phosphorylation. This observation supports suggestion that in this cell type sustained intracellular acidosis-mediated NHE1 activation may be masked somewhat by other basal stimulatory effects that result in basal NHE1 phosphorylation.

Overall we can conclude that sustained intracellular acidosis stimulates NHE1 activation via a phosphorylation-dependent mechanism in a MEK-dependent pathway that is significant and reproducible.

1.3. Serine and threonine residues in the NHE1 cytoplasmic tail

To identify specific residues that could be involved in the sustained intracellular acidosis activation of NHE1 a series of serine/threonine to alanine mutations were made. The selection of residues was based on several lines of evidence. Residues Ser⁶⁹³, Ser⁷⁶⁶, Ser⁷⁷⁰, Thr⁷⁷⁹, and Ser⁷⁸⁵ were selected for mutation as a result of a study completed by our laboratory in collaboration with the laboratory of Dr. Liang Li of the Chemistry Department at the University of Alberta (75). Additionally, Ser⁷⁷¹ was chosen due to its proximity to residues Ser⁷⁶⁶ and Ser⁷⁷⁰. In this study open tubular immobilized metal ion affinity

chromatography (OT-IMAC) combined with matrix-assisted laser desorption/ionization (MALDI) tandem mass spectrometry (MS) was used to identify ERK2 phosphorylation sites on NHE1. Following *in vitro* NHE1 phosphorylation by ERK2, the protein samples were digested with trypsin, and then digested further with endoproteinase Asp-N if the phosphorylation sites could not be assigned. The OT-IMAC method was used to separate and enrich the protein digest mixtures before subjecting the samples to the MALDI tandem-MS.

Mutation of residues Thr⁷¹⁸, Ser⁷²³, Ser⁷²⁶, and Ser⁷²⁹ was based on results of another study in which mass spectroscopy and deletion analysis of NHE1 identified the residues as being important in activation of NHE1 following trophic factor withdrawal (59). This study demonstrated that p38 mediated the *in vitro* phosphorylation of the above stated residues and the *in vivo* stimulation of NHE1 following trophic factor withdrawal

The residues selected for mutation were completed as groups rather than single residue mutations, with the exception of Ser⁶⁹³. Much of our motive for this approach came from other observations made about phosphorylated amino acids in the cystic fibrosis transmembrane regulator (CFTR) protein. The CFTR protein is a large Cl⁻ channel that, when defective, causes the cystic fibrosis disease state. This protein has a regulatory domain that is targeted for phosphorylation resulting in both stimulatory and inhibitory regulation of the protein (150). It has been demonstrated in the CFTR protein, that for certain residues to mediate their

regulatory effect in response to phosphorylation they are dependent on the simultaneous phosphorylation of other residues (10). This is not the result of an additive effect, but rather requires activation of groups of phosphorylatable residues. Due to this observation, we selected groups of residues for mutation, anticipating the possibility that a similar effect could be observed in the Na^+/H^+ exchanger isoform 1. Additionally, mutating groups of residues allowed us to look at numerous residues in an efficient manner, and identify important groups of residues that could be studied individually in future projects. Figure 13 identifies the specific mutated residues, and the groups of mutations.

Mutation of the selected residues did not have large effects on the normal functioning of the NHE1 protein. Figure 4 illustrates the expression levels of the wildtype NHE1 and each of the mutant NHE1 proteins expressed in AP-1 cells, additionally it shows that no HA-tagged NHE1 is expressed in the untransfected AP-1 cells. From this set of results it is clear that there are variations in the level of protein expressed for each mutant relative to the wildtype protein, but the key result was that the mutations did not "knockout" the protein. A significant and measurable level of expression was observed for each mutant, and further analysis of the mutant proteins proved that a significant amount of the expressed protein was being properly targeted to the plasma membrane (Figure 5). Finally, evaluation of the functional state of the mutated proteins proved that the mutations did not abolish Na^+/H^+ exchange ability as demonstrated by cells

recovery after an acute NH_4Cl induced acid load (Figure 6). We can conclude from this set of experiments that the mutations introduced into the NHE1 protein do not cause severe detrimental effects to the functional state of the protein, and therefore the mutant proteins can be used to further study the role of specific residues in NHE1 stimulatory mechanisms.

1.4. Role of specific residues in NHE1 activation

The sustained intracellular acidosis method of NHE1 stimulation was used to assess the effect of specific mutations in the cytosolic region on NHE1 activation. Figure 11 displays the results of control and stimulatory assays completed for the wildtype and mutant NHE1 proteins expressed in AP-1 cells. The single $\text{Ser}^{693} \rightarrow \text{Ala}$ mutant, and the quadruple $\text{Thr}^{718} \rightarrow \text{Ala}$ and $\text{Ser}^{723/26/29} \rightarrow \text{Ala}$ mutant had no effect on the stimulatory response of NHE1 to sustained intracellular acidosis treatment. This suggests that these residues are not involved in this stimulatory mechanism, and therefore they were not pursued further.

On the other hand, both the triple $\text{Ser}^{766/70/71} \rightarrow \text{Ala}$ and double $\text{Thr}^{779} \rightarrow \text{Ala}$ and $\text{Ser}^{785} \text{Ala}$ mutants resulted in a reduced stimulatory effect relative to the wildtype protein. The $\text{Ser}^{766/70/71} \rightarrow \text{Ala}$ triple mutant completely abolished any significant stimulatory effect due to sustained intracellular acidosis, whereas the $\text{Thr}^{779} \rightarrow \text{Ala}$ and $\text{Ser}^{785} \text{Ala}$ double mutant still demonstrated some level of

stimulatory effect that was reduced from that observed for the wildtype but not significantly different. Based on these results we can predict that residues Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ are important in the sustained intracellular acidosis mechanism of NHE1 activation, and that there is a possible involvement of Thr⁷⁷⁹ and Ser⁷⁸⁵.

To determine whether residues Ser⁷⁶⁶, Ser⁷⁷⁰, Ser⁷⁷¹, Thr⁷⁷⁹, and Ser⁷⁸⁵ were involved in the phosphorylation-dependent activation of NHE1 via the ERK2/p90^{RSK} pathway NHE1 phosphorylation levels were assessed after control or stimulatory treatment of cells. Sustained intracellular acidosis treatment of cells resulted in an increase in NHE1 phosphorylation above the basal level observed in the control treated cells. Figure 12 shows the results for AP-1 cells expressing wildtype NHE1, the triple Ser^{766/770/771} mutant, or the double Thr⁷⁷⁹-Ser⁷⁸⁵ mutant. In addition to abolishing the stimulatory effect of sustained acidosis on NHE1 activity (Figure 11) mutation of Ser^{766/770/771} prevented the increase in NHE1 phosphorylation observed for the wildtype protein. Mutation of Thr⁷⁷⁹-Ser⁷⁸⁵ did not significantly alter the phosphorylation state relative to the wildtype protein. These results confirm that residues Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ are involved in the phosphorylation-dependent activation of NHE1 via the ERK2/p90^{RSK} pathway. In the absence of these residues NHE1 is not phosphorylated in response to sustained intracellular acidosis treatment, implicating one or all of these residues as being specifically phosphorylated by the activated kinases resulting in the activation of NHE1.

Although *in vitro* work had identified residues Thr⁷¹⁸, Ser⁷²³, Ser⁷²⁶, and Ser⁷²⁹ as being phosphorylated (59), we did not see any negative effect on NHE1 stimulation via sustained intracellular acidosis when the residues were mutated to alanine (Figure 11). There are several possible reasons for this difference including different cells types being used. Khalad *et al* stimulated NHE1 activity in mouse B-cells whereas we activated the exchanger in Chinese hamster ovary cells. Additionally, they used trophic factor withdrawal to stimulate NHE1 while we used sustained intracellular acidosis, which has been shown to activate the ERK kinase pathway by our group and others (50). Trophic factor withdrawal resulted in only p38 kinase activation (59). These differences in cell type, stimulatory method, and pathway activation are likely the source of the differences.

Recently our laboratory has identified another residue in the C-terminal tail of NHE1 that is potentially phosphorylated by ERK2. When identifying the site of CAII interaction as residues 790-802 of NHE1 Li *et al* also identified Ser⁷⁹⁶ as a potential phosphorylation target (71). ERK2 was capable of phosphorylating the residue *in vitro*, and mutation of the residue to alanine resulted in a reduction of CAII binding and NHE1 activation *in vivo*.

2. Summary and conclusions

The human Na^+/H^+ exchanger isoform 1, when expressed in AP-1 cells, is activated by sustained intracellular acidosis. This mechanism of activation involves initiating the classical ERK1/2 mitogen activated protein kinase signaling cascade resulting in ERK2 and p90^{RSK} kinase activation. The NHE1 stimulatory effect is dependent on ERK2 and p90^{RSK} activation, and results in an increase in phosphorylation above the basal level observed in control treated cells. If residues Ser^{766} , Ser^{770} , and Ser^{771} are mutated to Ala the sustained intracellular acidosis mediated increase in NHE1 phosphorylation and resulting increase in Na^+/H^+ exchange activity is abolished. This implicates Ser^{766} , Ser^{770} , and Ser^{771} as being the residues specifically phosphorylated in response to this stimulatory method.

Considering all of the results, we can propose a mechanism by which sustained intracellular acidosis mediates NHE1 activation (Figure 14). The increase in intracellular acidity initiates Raf mediated MEK1 activation. Activated MEK1 phosphorylates and activates ERK2, which in turn activates p90^{RSK} . The ERK2 and p90^{RSK} kinases mediate the phosphorylation of one or all of the following residues: Ser^{766} , Ser^{770} , and Ser^{771} . Phosphorylation of one or all of these residues alters the configuration of the NHE1 cytoplasmic tail, modifying the interaction of the regulatory tail domain with the ion transport transmembrane

domain. This change in the association between the two domains alters the rate at which the exchanger can mediate Na^+/H^+ exchange.

The conclusions of this study are:

- I. Human NHE1 expressed in AP-1 cells is activated by sustained intracellular acidosis via a phosphorylation-dependent ERK2/p90^{RSK} mechanism
- II. NHE1 residues Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ are critical residues in the sustained intracellular acidosis method of NHE1 activation
- III. Phosphorylation of Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ occurs in response to sustained intracellular acidosis
- IV. Phosphorylation of Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ is required for the sustained intracellular acidosis activation of NHE1

3. Future experiments

- I. Currently the mutants designed and described in this report are being introduced into an adenoviral expression system that will be utilized to infect primary cell cultures of rat cardiomyocytes. This will allow our laboratory to further analyze the role of specific residues in regulation of the Na^+/H^+ exchanger in the myocardium. Our interest in studying NHE1 in the myocardium is due to its significant role in the pathological process of heart disease. Additionally, signaling pathways are not completely conserved between tissues so it is important to see how the signaling varies from our studies in AP-1 cells to that of cardiomyocytes.
- II. These mutants can be used to analyze other NHE1 stimulatory pathways. Stimulation of pathways that result in the activation of different kinases from those studied within this report could lead to the identification of residues critical for distinct modes of NHE1 activation.
- III. This report identified residues Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ as being critical for sustained intracellular acidosis-mediated NHE1 activation. It is necessary to determine the individual involvement of each residue in this mechanism. By developing single and double mutants and assessing the effect on NHE1 stimulation, the involvement of the individual residues can be determined. This will also allow us to establish whether the

residues act in concert as observed for the CFTR protein (10), or independently. By this I mean that phosphorylation of two or more sites are required for the stimulatory effect to occur rather than each site providing a portion of the stimulatory effect in an additive manner.

- IV. More work is required to delineate the signaling cascade activated by sustained intracellular acidosis. From our studies and those completed by Haworth *et al* (50) it is clear that the ERK1/2 pathway is stimulated via Raf and MEK1 activation, but it is still unclear what upstream events are occurring to initiate this cascade.
- V. The phosphorylation mutants can also be used to analyze other NHE1 stimulatory pathways. This could potentially identify residues important for distinct mechanisms of NHE1 regulation.

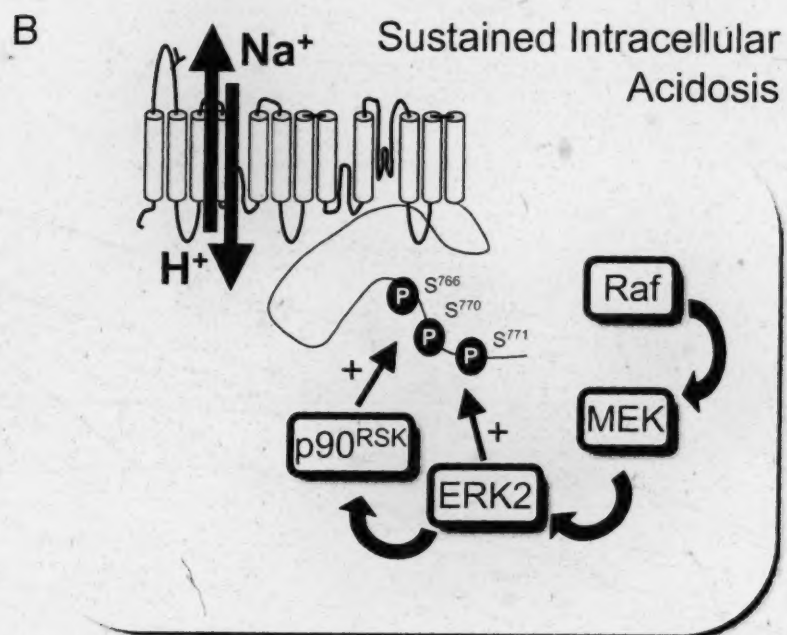
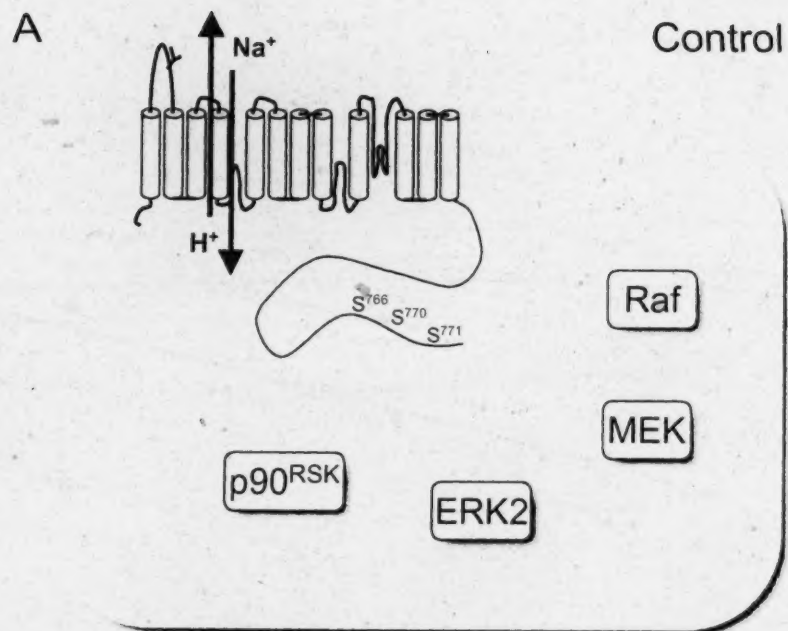
Figure 13. C-terminal 181 residues of NHE1

The bold residues indicate Ser/Thr residues mutated to Ala residues. The groups of mutated residues are underlined.

634	MILRNNLQKT	RQRLRSYNRH	TLVADPYEEA	WNQMLLRQK	372
673	ARQLEQKINN	YLTYP AHKLD	<u>S</u> PTMSRARI	SDPLAYEPKE	712
713	DLPVITIDPA	<u>SPQSPES</u> VDL	VNEELKGKVL	GLSRDPAKVA	752
753	EEDEDDDDGGI	MMR <u>SKET</u> SSP	GTDDVFTTAP	<u>SDSPSSQ</u> RIQ	792
793	RCLSPRGPH	EPGEGEPFFP	KGQ	815	

Figure 14. Sustained intracellular acidosis mechanism of NHE1 activation.

A: Depicts the cellular events occurring during the control treatment of cells. B: Depicts the events occurring during the sustained intracellular acidosis treatment of cells. In controls the Raf→MEK→ERK2→p90^{RSK} pathway is not activated, Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ are not phosphorylated, and Na⁺/H⁺ exchange activity is not stimulated. In the sustained intracellular acidosis treated cells, the Raf→MEK→ERK2→p90^{RSK} pathway is activated, Ser⁷⁶⁶, Ser⁷⁷⁰, and Ser⁷⁷¹ are phosphorylated, and Na⁺/H⁺ exchange activity is stimulated.



Chapter V

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